PATENT COOPERATION TR[illegible]TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark
Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 04 October 2000 (04.10.00)	
International application No. PCT/CA00/00147	**Applicant's or agent's file reference** DH/12987.5
International filing date (day/month/year) 11 February 2000 (11.02.00)	**Priority date** (day/month/year) 11 February 1999 (11.02.99)
Applicant DESGROSEILLERS, Luc et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

06 September 2000 (06.09.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer F. Baechler
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

REC'D 08 MAY 2001
WIPO PCT

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference KB/12987.5	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/CA00/00147	International filing date *(day/month/year)* 11/02/2000	Priority date *(day/month/year)* 11/02/1999
International Patent Classification (IPC) or national classification and IPC C12N15/57		
Applicant UNIVERSITE DE MONTREAL et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☒ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☒ Certain documents cited
   VII ☒ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 06/09/2000	Date of completion of this report 04.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer Montero Lopez, B Telephone No. +31 70 340 3739

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00147

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-32	as originally filed			

Claims, No.:

1-38	as received on	13/04/2001	with letter of	11/04/2001

Drawings, sheets:

1/20-20/20	as originally filed			

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 29.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☒ the claims, or said claims Nos. 29 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

☒ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	2, 5-7
	No:	Claims	
Inventive step (IS)	Yes:	Claims	2, 5-7
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	2, 5-7
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I
Basis of the report

The amendments filed with the International Bureau under Article 19(1) introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 19(2) PCT. The amendments concerned are the following:

1. Claim 1: The application as filed does not disclose a method for obtaining a Neprilysin-like metallopeptidase as claimed in claim 1. The application discloses in pages 14-16 a particular method for the cloning and recombinant expression of mouse NL1 of which claim 1 constitutes an undue generalization.

2. Claims 3, 4, and 5-7 as far as dependent on claims 3 and 4: A soluble metallopeptidase sharing about 80% homology with the C-terminus of the furin site shown in Figure 3 has not been specifically disclosed in the application. Soluble forms of NL1 and NL3, consisting of the ectodomain are referred to in page 10. This does not provide support for soluble enzymes sharing 80% homology with the disclosed soluble forms.

3. Claims 8 and 22: The application as filed does not disclose such a general method for obtaining a substrate of a metallopeptidase. Page 24 discloses several methods for identifying substrates, of which claims 8 and 22 constitute an undue generalization.

4. Claims 9-12 and 23-26: Page 25 discloses a method for identifying inhibitors. The method of claims 9 and 23 constitutes an undue generalization of the subject-matter disclosed in page 25. No support exists in the application for inhibitors or uses thereof other than the speculative statement referred to in page 25.

5. Claims 13 and 27: The direct of use of the metallopeptidase to manage disease is not disclosed in the application. Page 27 discloses merely that it is possible to help the patient by managing the activity of the enzyme.

6. Claims 14, 15 and 28: The application discloses a sequence encoding the N-terminal part up to the furin-recognition sequence in figure 3 (see page 10), but not a sequence

having 80% homology to it.

7. Claims 16-21: A metallopeptidase sharing about 80% homology with the C-terminus of the furin site shown in Figure 4 has not been specifically disclosed in the application. Soluble forms of NL1 and NL3, consisting of the ectodomain are referred to in page 10. This does not provide support for soluble enzymes sharing 80% homology with the disclosed soluble forms.

8. Claims 30-38: A metallopeptidase sharing about 80% homology with the C-terminus of the transmembrane domain shown in Figure 5 has not been specifically disclosed in the application. Soluble forms of NL1 and NL3, consisting of the ectodomain are referred to in page 10. This does not provide support for soluble enzymes sharing 80% homology with the disclosed soluble forms, neither to any of its applications.

Re Item IV

Lack of unity of invention

The present application relates to endopeptidase-like metallopeptidases and a method for obtaining. Polypeptides belonging to the neutral metallopeptidase family have been already been described in the state of the art (see pages 1 and 2 of the description). The article "Gene" 1996, vol.174, pages 135-143 discloses the cloning of a metallopeptidase of the neutral endopeptidase family. In the light of the prior art a problem underlying the present application can be formulated as providing further endopeptidase-like metallopeptidases. The following solutions are proposed:

1. A polypeptide of the sequence disclosed in figure 3, fragments and variants thereof and corresponding nucleic acid (Claims 2-15).

2. A polypeptide of the sequence disclosed in figure 4, fragments and variants thereof and corresponding nucleic acid (Claims 16-27).

3. A polypeptide of the sequence disclosed in figure 5, fragments and variants thereof and corresponding nucleic acid (Claims 30-38).

A further problem identified in the application relates to a method for obtaining a Neprilysin-like metallopeptidase. The solution proposed as formulated in claim 1 is as follows:

1. Using primers in the C- and N-terminus of the sequence His-Glu-Xaa-Xaa-His sequence in a PCR method.

Given the essential difference between the problems posed and their corresponding solutions, since neutral endopeptidase-like metallopeptidases have already been disclosed in the state of the art, and due to the differences in primary sequence between the polypeptides disclosed as solutions to the first problem, as well as among their corresponding nucleic acid sequences and since in the light of the state of the art, no other technical feature could be distinguished as being new and common to the identified problems and corresponding solutions, the IPEA is of the opinion that there is no single inventive concept underlying the plurality of the claimed inventions in the present application, in the sense of Rule 13.1 PCT.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following document:

D1: US-A-5 688 640 (MASASHI YANAGISAWA) 18 November 1997 (1997-11-18)

1. Claims 2 and claims 5-7, as far as dependent on claim 2 relate to a metallopeptidase sharing about 80% homology with the amino acid sequence of figure 3. No such sequence has been disclosed in the state of the art and therefore, claims 2 and 5-7 are novel and comply with the requirements of Article 33(2) PCT.

2. Document D1 discloses the aminoacid and nucleic acid sequences of the Endothelin Converting Enzyme ECE-1 which is a membrane-bound neutral metalloprotease

expressed in endothelial cells, with a sequence significantly different from the one disclosed in figure 3 of the application. No hint exists in the state of the art which would allow the skilled person to retrieve a metallopeptidase with 80% homology to the one disclosed in figure 3. Consequently, the subject-matter of claims 2 and 5-7 involves an inventive step and meets the requirements of Article 33(3) PCT.

Re Item VI
Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
PCT/FR99/00807	21/10/1999	07/04/1999	08/04/1998

Re Item VII
Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the document D1 is not mentioned in the description, nor is this document identified therein.

2. Claim 2 contains a reference to the drawings. According to Rule 6.2(a) PCT, claims should not contain such references except where absolutely necessary, which is not the case here.

Re Item VIII
Certain observations on the international application

1. The term "about" used in claim 2 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claim unclear (Article 6 PCT).

WHAT IS CLAIMED IS:

1. A method for obtaining a Neprilysin-like (NEP-like) metallopeptidase which comprises the following steps:
   - selecting a primer in C-terminus of the His-Glu-Xaa-Xaa-His (where Xaa represents any amino acid) with a degenerate nucleotide sequence complementary to at least the Gly-Glu-Asn-Ile-Ala-Asp amino acid sequence of known NEP-like metallopeptidases with sufficient binding capacity;
   - selecting a primer in N-terminus of the His-Glu-Xaa-Xaa-His (where Xaa represents any amino acid) with a degenerate nucleotide sequence complementary to a conserved amino acid sequence with preferably 80% homology with known NEP-like metallopeptidases and sufficient binding capacity;
   - contacting said primer with tissue nucleic acids to yield PCR products;
   - selecting said PCR products that contain the His-Glu-Xaa-Xaa-His motif; and
   - completing the sequence of said selected PCR products with standard methods.
2. A metallopeptidase sharing about 80% homology with the amino acid sequence shown in Figure 3.
3. A metallopeptidase which is soluble sharing about 80% homology with the amino acid sequence in C-terminus of the furin site shown in Figure 3.
4. A metallopeptidase which is soluble sharing about 80% homology with the amino acid sequence shown in Figure 3 and with an enzymatic activity capable of degradation of known Neprilysin substrates, preferably Tyrosyl-[3,5-^{3}H1)(D-Ala$_2$)-Leu$_5$-enkephalin and bradykinin.
5. A composition comprising a metallopeptidase as defined in any one of claims 2-4.

6. A nucleic acid encoding a metallopeptidase as defined in any one of claims 2-4.

7. An antibody directed against a metallopeptidase as defined in any one of claims 2-4.

8. A method for obtaining a substrate of a metallopeptidase as defined in any one of claims 2-4, which comprises the steps of:

-- contacting said metallopeptidase with a molecule or extract; and

-- assaying the resulting solution for a decrease in said molecule or extract, when compared with the same but in absence of said metallopeptidase, as an indication of the presence of said substrate.

9. A method for obtaining an inhibitor of a metallopeptidase as defined in any one of claims 2-4, which comprises the steps of:

-- contacting said metallopeptidase with a molecule or extract in the presence of a substrate selected known NEP substrates, preferably Tyrosyl-[3,5-^{3}H1)(D-Ala$_2$)-Leu$_5$-enkephalin and bradykinin; and

-- assaying the resulting solution for an increase in said substrate, when compared with the same but in absence of said metallopeptidase, as an indication of the presence of said inhibitor.

10. An inhibitor obtained from the method of claim 9.

11. The use of a known NEP inhibitor or an inhibitor as defined in claim 10 to control the enzymatic activity of a metallopeptidase as defined in any one of claims 2-4.

12. The use of a known NEP inhibitor or an inhibitor as defined in claim 10 to manage disease relating to the physiological status of the cardiovascular system, the central nervous system, the spleen, the liver, the kidney, the male reproductive system or the maturation of spermatozoa.

13. The use of a metallopeptidase as defined in any one of claims 2-4 to manage disease relating to the physiological status of the cardiovascular

system, the central nervous system, the spleen, the liver, the kidney, the male reproductive system or the maturation of spermatozoa.

14. A recombinant vector comprising 80% homology with the nucleic acid encoding the N-terminal part of the amino acid sequence shown in Figure 3, which N-terminal part terminates with a furin-recognition sequence.

15. A method for producing a soluble form of a protein, polypeptide or part thereof which comprises:

- obtaining nucleic acids encoding said protein, polypeptide or part thereof;
- fusing said nucleic acids in phase with an N-terminal fragment wherein said N-terminal fragment comprises a cleavable furin-like site located in C-terminus past the transmembrane region or is an N-terminal part as defined in claim 14;
- having the fused nucleic acids to be expressed in a host cell which expresses or is made to express furin in the presence of a culture medium; and
- recovering said soluble form in the culture medium.

16. A protein, polypeptide or part thereof produced by the method defined in claim 15, wherein said protein, polypeptide or part thereof is a metallopeptidase sharing about 80% homology with the region in C-terminus of the putative furin site of the amino acid sequence shown in Figure 4.

17. A metallopeptidase sharing about 80% homology with the region in C-terminus of the putative furin site of the amino acid sequence shown in Figure 4.

18. A metallopeptidase sharing about 80% homology with the region in C-terminus of the putative furin site of the amino acid sequence shown in Figure 4 and with an enzymatic activity capable of degradation of known

NEP substrates, preferably Tyrosyl-[3,5-^{3}H1)(D-Ala$_2$)-Leu$_5$-enkephalin and bradykinin.

19. A composition comprising a metallopeptidase as defined in any one of claims 16-18.

20. A nucleic acid encoding a metallopeptidase as defined in any one of claims 16-18.

21. An antibody directed against a metallopeptidase as defined in any one of claims 16-18.

22. A method for obtaining a substrate of a metallopeptidase as defined in any one of claims 16-18, which comprises the steps of:

-- contacting said metallopeptidase with a molecule or extract; and

-- assaying the resulting solution for a decrease in said molecule or extract, when compared with the same but in absence of said metallopeptidase, as an indication of the presence of said substrate.

23. A method for obtaining an inhibitor of a metallopeptidase as defined in any one of claims 16-18, which comprises the steps of:

-- contacting said metallopeptidase with a molecule or extract in the presence of a substrate selected from known NEP substrates or a protein, polypeptide or part thereof produced by the method of claim 15, preferably Tyrosyl-[3,5-^{3}H1)(D-Ala$_2$)-Leu$_5$-enkephalin and bradykinin; and

-- assaying the resulting solution for an increase in said substrate, when compared with the same but in absence of said metallopeptidase, as an indication of the presence of said inhibitor.

24. An inhibitor obtained from the method of claim 23.

25. The use of a known NEP inhibitor or an inhibitor as defined in claim 24 to control the enzymatic activity of a metallopeptidase as defined in any one of claims 16-18.

26. The use of a known NEP inhibitor or an inhibitor as defined in claim 24 to manage disease relating to the physiological status of the cardiovascular system, the central nervous system, the spleen, the liver, the kidney, the male reproductive system or the maturation of spermatozoa.

27. The use of a metallopeptidase as defined in any one claims 16-18 to manage disease relating to the physiological status of the cardiovascular system, the central nervous system, the spleen, the liver, the kidney, the male reproductive system or the maturation of spermatozoa.

28. A method as defined in claim 15, wherein said protein, polypeptide or part thereof is beta-endorphin.

29. A recombinant host cell capable of expressing a protein, polypeptide or part thereof transplanted in a mammal to manage a disease, physiological process or pain.

30. A metallopeptidase sharing about 80% homology with the amino acid sequence located in the C-terminus of the predicted transmembrane domain of the amino acid sequence shown in Figure 5 which has been produced by the method of claim 15, by fusing in frame a cleavable signal peptide in N-terminus of said amino acid sequence or by transforming said predicted transmembrane domain into a cleavable signal peptide.

31. A composition comprising a metallopeptidase as defined in claim 30.

32. An antibody directed against a metallopeptidase as defined in claim 30.

33. A method for obtaining a substrate of a metallopeptidase as defined in claim 30, which metallopeptidase shares about 80% homology with the C-terminal region of the predicted transmembrane domain of the amino acid sequence shown in Figure 5, comprising the steps of:

-- contacting said metallopeptidase with a molecule or extract; and

-- assaying the resulting solution for a decrease in said molecule or extract, when compared with the same but in absence of said metallopeptidase, as an indication of the presence of said substrate.

34. A method for obtaining an inhibitor of a metallopeptidase sharing about 80% homology with the C-terminal region of the predicted transmembrane domain of the amino acid sequence shown in Figure 5, which comprises the steps of:

-- contacting said metallopeptidase with a molecule or extract in the presence of a substrate produced by the method of claim 33; and

-- assaying the resulting solution for an increase in said substrate, when compared with the same but in absence of said metallopeptidase, as an indication of the presence of said inhibitor.

35. An inhibitor obtained by the method of claim 34.

36. The use of an inhibitor as defined in claim 35 to control the enzymatic activity of the metallopeptidase sharing about 80% homology with the C-terminal region of the predicted transmembrane domain of the amino acid sequence shown in Figure 5.

37. The use of an inhibitor as defined in claim 35 to manage disease relating to the physiological status of the central nervous system, the spleen or the bones.

38. The use of a metallopeptidase as defined in claim 30 to manage disease relating to the physiological status of the cardiovascular system, the central nervous system, the spleen or the bones.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference DH/12987.5	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/CA 00/00147	International filing date *(day/month/year)* 11/02/2000	(Earliest) Priority Date *(day/month/year)* 11/02/1999
Applicant UNIVESITE DE MONTREAL et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      - ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :
      - ☐ contained in the international application in written form.
      - ☐ filed together with the international application in computer readable form.
      - ☒ furnished subsequently to this Authority in written form.
      - ☒ furnished subsequently to this Authority in computer readble form.
      - ☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      - ☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☒ **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**
   - ☐ the text is approved as submitted by the applicant.
   - ☒ the text has been established by this Authority to read as follows:

   METALLOPROTEASES OF THE NEPRILYSIN FAMILY

5. With regard to the **abstract,**
   - ☐ the text is approved as submitted by the applicant.
   - ☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 10
   - ☐ as suggested by the applicant.
   - ☐ because the applicant failed to suggest a figure.
   - ☒ because this figure better characterizes the invention.
   - ☐ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 00/00147

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1, 5-8, 11-22 partially, and 2

   Neutral endopeptidase-like metallopeptidase of figure 3, nucleic acid encoding it, vector and host cell comprising the same and use thereof for producing the metallopeptidase; oligonucleotides and antibodies thereof as well as their use for detecting the metallopeptidase in a sample; vector comprising a sequence encoding the N-terminal part of the metallopeptidase and use thereof for producing a soluble form of a protein of interest; soluble metallopeptidase

2. Claims: 1, 5-8, 11-22 partially, 3

   Neutral endopeptidase-like metallopeptidase of figure 4, nucleic acid encoding it, vector and host cell comprising the same and use thereof for producing the metallopeptidase; oligonucleotides and antibodies thereof as well as their use for detecting the metallopeptidase in a sample; vector comprising a sequence encoding the N-terminal part of the metallopeptidase and use thereof for producing a soluble form of a protein of interest; soluble metallopeptidase

3. Claims: 1, 5-8, 11, 12, 17-22 partially, 4

   Neutral endopeptidase-like metallopeptidase of figure 5, nucleic acid encoding it, vector and host cell comprising the same and use thereof for producing the metallopeptidase; oligonucleotides and antibodies thereof as well as their use for detecting the metallopeptidase in a sample; soluble metallopeptidase

4. Claims: 9, 10

   Method for screening molecules related to neutral endopeptidase by using consensus sequences on either side of a His-Glu-Xaa-Xaa-His sequence

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

Line 2 of the text:

Please delete the word "new".

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00147

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/57 C12N9/64 C12N15/85 C12N5/10 C12Q1/68
C12N15/62 C07K16/40 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12Q C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, MEDLINE, STRAND, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 688 640 A (MASASHI YANAGISAWA) 18 November 1997 (1997-11-18) column 2, line 65 -column 6, line 21 column 11, line 4 -column 14, line 35 column 14, line 64 -column 15, line 14 column 17, line 8 -column 18, line 20; examples 1-4 --- -/--	1-8, 11-22

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00147

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	SCOTT I C ET AL: "Molecular cloning, expression and chromosomal localization of a human gene encoding a 33 kDa putative metallopeptidase (PRSM1)" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES,GB,ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 174, no. 1, 26 September 1996 (1996-09-26), pages 135-143, XP004043253 ISSN: 0378-1119 the whole document ---	1-8, 11-22
X	NIGEL M. HOOPER: "Families of Zinc metalloproteases" FEBS LETTERS, vol. 354, no. 1, 31 October 1994 (1994-10-31), pages 1-6, XP002147067 AMSTERDAM NL cited in the application page 1, right-hand column, paragraph 2 page 1, right-hand column, last paragraph -page 2, left-hand column, paragraph 1 page 5, right-hand column, paragraph 1 ---	9,10
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P,X	KOJI IKEDA ET AL.: "Molecular identification and characterization of novel membrane-bound metalloprotease, the soluble secreted form of which hydrolyzes a variety of vasoactive peptides" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 45, 5 November 1999 (1999-11-05), pages 32469-32477, XP002140284 MD US cited in the application abstract page 32469, left-hand column, paragraph 1 -right-hand column, paragraph 1 page 32470, left-hand column, paragraph 3 page 32470, left-hand column, last paragraph -right-hand column, paragraph 1 page 32470, right-hand column, paragraph 3 page 32471, left-hand column, paragraph 4 -right-hand column, paragraph 4; figure 1 page 32474, right-hand column, last paragraph -page 32476, right-hand column, paragraph 2 --- -/--	1,5-9, 17-20,22

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	VALDENAIRE O ET AL: "XCE, A NEW MEMBER OF THE ENDOTHELIN-CONVERTING ENZYME AND NEUTRAL ENDOPEPTIDASE FAMILY, IS PREFERENTIALLY EXPRESSED IN THE CNS" MOLECULAR BRAIN RESEARCH,NL,ELSEVIER SCIENCE BV, AMSTERDAM, vol. 64, no. 2, 1999, pages 211-221-221, XP000863086 ISSN: 0169-328X abstract page 211, left-hand column, paragraph 1 -page 212, right-hand column, paragraph 1 page 212, right-hand column, last paragraph -page 213, left-hand column, paragraph 1 page 215, left-hand column, paragraph 2 -page 216, right-hand column, paragraph 1; figure 1 page 219, left-hand column, paragraph 2 page 219, right-hand column, paragraph 2 -page 220, left-hand column, paragraph 1	1,4-9, 19,20,22
X	& DATABASE EMBL 'Online! Accession number Y16187, 7 January 1999 (1999-01-07) VALDENAIRE, O.: "Homo sapiens mRNA for XCE protein" the whole document -----	1,4-8, 17-22

INTERNATIONAL SEARCH REPORT

mation on patent family members

ernational Application No
PCT/CA 00/00147

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5688640 A	18-11-1997	NONE	
WO 9953077 A	21-10-1999	FR 2777291 A	15-10-1999

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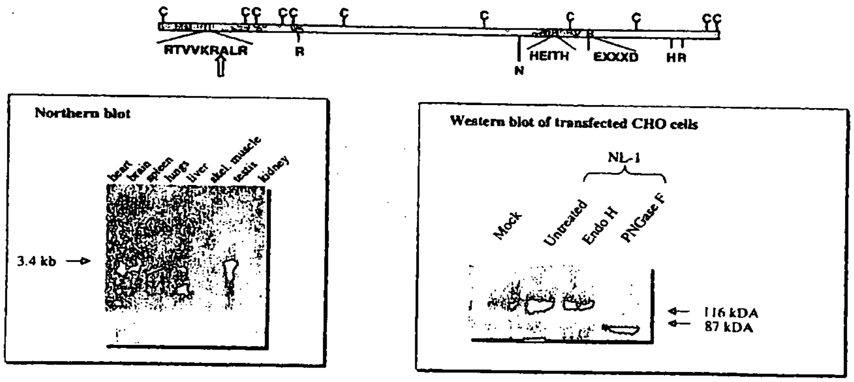
(54) Title: NEW METALLOPROTEASES OF THE NEPRILYSIN FAMILY

Structure and expression of NL-1



(57) Abstract

In this paper, we describe RT-PCR strategies that allowed us to identify and clone members of the NEP-like family. Degenerate oligoncleotide primers corresponding to consensus sequences located on either side of the HEXXH consensus sequence for zincins were designed and used in RT-PCR with mouse and human testis cDNAs. DNA fragments with lengths expected from the sequence of this class of enzympes were obtained. These DNA fragments were cloned and sequenced. Using this PCR strategy and the PCR fragments as probes to screen cDNA libraries, three zincin-like peptidases were identified in addition of known members of the family. The cDNA sequences allowed to derive specific probes for Northern and *in situ* hybridization, and probe human chromosomes to localize the gene and establish potential links to genetic diseases. Furthermore, these cDNA sequences were used to produce recombinant fusion proteins in *Escherichia coli* in order to raise specific antibodies. Finally, the cDNA sequences were cloned in mammalian expression vectors and transfected in various mammalian cell lines to produce active recombinant enzymes suitable for testing specific inhibitors.

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TITLE OF THE INVENTION

New Metalloproteases of the Neprilysin Family

BACKGROUND OF THE INVENTION

Peptides are used by cells from yeast to mammals to elicit physiological responses. The use of peptides as messengers usually involves the following steps: 1) production and release of the peptide by a specific cell, 2) interaction of the peptide with a receptor on the surface of the target cell, and 3) degradation of the peptide to terminate its action. The first and last steps of this scheme require the participation of proteases/peptidases. There is increasing evidence that membrane-associated zinc-metallopeptidases play important roles in both of these steps. Although activation of prohormone precursors into bioactive peptides is generally performed by proteases of the subtilisin family located in the *Trans*-Golgi Network or in secretory granules of the cell (for a review see: (Seidah and Chrétien, 1995)) a few peptides need a final processing step. This step involves the action of membrane-associated zinc-metallopeptidases. Two cases are particularly well documented: angiotensin-converting enzyme (ACE) which cleaves inactive angiotensin I into angiotensin II (Corvol and Williams, 1997) and endothelin-converting enzymes (ECEs) which cleave isoforms of big endothelins into endothelins (Turner, 1997a). In addition to their role in peptide activation, cell surface zinc-metallopeptidases have also been implicated in the termination of the peptidergic signal by breaking down the active peptides into inactive fragments. One of the best known of these peptidases is probably Neutral Endopeptidase-24.11 (Neprilysin, NEP) that has been implicated in the physiological degradation of several bioactive peptides (Kenny, 1993). Interestingly, NEP and the ECEs show significant structural similarities and appear to be members of a family of peptidases that also includes PEX, a newly discovered and not yet characterized peptidase, and the KELL blood group protein (Turner and Tanzawa, 1997b). Because of their important role as regulators of bioactive peptide activity, these enzymes (more specifically NEP and the ECEs) have been identified as putative targets for therapeutic intervention, similar to the way ACE inhibitors are used to control blood pressure. The recent discovery of PEX, another member of the family, which appears to be involved in phosphate homeostasis, raised the possibility that other yet unknown members might exist.

Members of the NEP-like family are type II membrane proteins consisting of three distinct domains: a short NH2-terminal cytosolic sequence, a single transmembrane region, and a large extracellular or ectodomain responsible for the catalytic activity of the enzyme. There are potential N-glycosylation sites and cysteine residues that are involved in disulfide bridges stabilizing the conformation of the active enzyme. These enzymes are metalloenzymes with a Zn atom in their active site. As such, they belong to the zincin family of peptidases which is characterized by the active site consensus sequence HEXXH (Hooper, 1994), where the two histidine residues are zinc ligands. In members of the NEP-like family of peptidases, the third zinc ligand is a glutamic acid residue located on the carboxy-terminus side of the consensus sequence. This characteristic puts them in the gluzincin sub-family (Hooper, 1994). The model enzyme for gluzincins is thermolysin (TLN) a bacterial protease whose 3D structure has been determined by X-ray crystallography (Holmes and Matthews, 1982). The active site of NEP has been extensively studied by site-directed mutagenesis and several residues involved in zinc binding (Devault et al., 1988b; Le Moual et al., 1991; Le Moual et al., 1994), catalysis (Devault et al., 1988a; Dion et al., 1993), or substrate binding (Vijayaraghavan et al., 1990; Beaumont et al., 1991; Dion et al., 1995; Marie-Claire et al., 1997) have been identified (for a recent review see Crine et al., 1997).

SUMMARY OF THE INVENTION

Here, we developed an RT-PCR strategy to look for other members of this important family of peptidases. This strategy allowed the molecular cloning and characterization of three additional NEP-like (NL) metallopeptidases (called NL-1, NL-2 and NL-3). Knowledge obtained through these studies allows the generation of reagents (nucleic acid probes and primers, antibodies and active recombinant enzymes) for further biochemical characterization of these enzymes and their pattern of expression and will greatly help the rational design of specific inhibitors that could be used as therapeutic agents.

Accordingly, the present invention relates to the following products:

A. Degenerate primers for screening new NEP-related enzymes;
B. NL-1, NL-2 and NL-3 proteins as NEP-related enzymes;
C. Nucleic acids encoding these enzymes;
D. Antibodies directed against the enzymes;
E. Recombinant vectors comprising the nucleic acids encoding the enzymes and hosts transformed therewith;
F. Fragments of the nucleic acids useful as probes or primers to hybridize and detect the presence of an NL-1, NL-2 and NL-3 genes, or to hybridize and amplify and produce gene fragments;
G. Soluble forms of NL-1, NL-2 and NL-3; and
H. Nucleic acids comprising the N-terminal part of NL-1 or NL-2 which terminates with a sequence encoding a furin recognition site, such nucleic acids being useful for making a fusion protein with the ectodomain of any protein of interest, and for releasing a soluble form of that protein of interest (containing the ectodomain) in the medium.

Also the present invention relates to the following methods:

A. A method for screening NEP-related enzymes that make use of degenerate primers or probes selected from a region of NEP family members in a highly conserved region, namely around the zinc-binding sites; and

B. A method for producing NL-1, NL-2 or NL-3 that includes the steps of culturing the above recombinant host and recovering NL-1, NL-2 and NL-3 gene products therefrom.

The present invention will be described hereinbelow by referring to specific embodiments and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

In the first section, general procedures leading to the identification and localization of NL-1, NL-2 and NL-3 are given. In the second section, slightly different procedures are given for completing or reiterating the work performed on NL-1.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Amino acid sequence comparison of human NEP, PEX, KELL and ECE1 peptidases. Amino acid sequences in boxes are those used to design the oligonucleotide primers. Numbers and arrows under the sequences identify the primer and its orientation.

Figure 2: Sequences of the oligonucleotide primers used in the PCR reactions.

Figure 3: Nucleotide and amino acid sequence of the mouse NL-1 cDNA. The sequence of the DNA fragment obtained by PCR is in brackets.

Figure 4: Partial nucleotide and amino acid sequence of the human NL-2 cDNA. The sequence of the DNA fragment obtained by PCR is in brackets.

Figure 5: Partial nucleotide and amino acid sequence of the human NL-3 cDNA.

Figure 6: Amino acid sequence comparison of NEP, NL-1, NL-2 and NL-3 peptidases.

Figure 7: *In situ* hybridization of mouse testis sections using NL-1 as a probe.

Figure 8: *In situ* hybridization of mouse sections using mouse NL-3 as a probe.

Figure 9: *In situ* hybridization of mouse spinal chord sections

Figure 10: Expression of NL-1 in mammalian cells.

Figure 11: Activity of recombinant soluble NL-1.

Figure 12: Expression of a soluble form of NL-3 using NL-1 amino-terminal domain.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

SECTION 1)

MATERIALS AND METHODS

DNA and RNA manipulations

All DNA manipulations and Northern blot analysis were performed according to standard protocols (Ausubel et al., 1988; Sambrook et al., 1989).

mRNA purification and cDNA synthesis

mRNAs were prepared from mouse testis using Quick Prep Micro mRNA purification kit (Pharmacia Biotech). Purified mRNAs were kept at -70° until ready used. First strand cDNA was synthesized from 1μg of mRNA using the First-Strand cDNA synthesis kit (Pharmacia Biotech). The human testis cDNA library was obtained from Clonetech.

Polymerase chain reaction protocol

PCR was performed in a DNA thermal cycler with 5 μl of cDNA template and 1 μl of Taq DNA polymerase in a final volume of 100 μl, containing 1 mM $MgCl_2$, 2 μM of each primer oligonucleotide, 20 μM of each dNTP and 5% DMSO. Cycling profiles included an initial denaturation step of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 40°C and 1.5 min at 72°C. A final extension step was performed at 72°C for 10 min. The amplified DNA was loaded on a 2% agarose gel and visualized by staining with ethidium bromide. Fragments ranging in size between 500-700 bp were cut and eluted from the gel. If needed, a second round of PCR was done with nested oligonucleotide primers, using 10 μl of the first PCR reaction, or of the eluted band cut from the agarose gel. Resulting fragments were ligated in pCR2.1 vector (Invitrogen) according to the distributor's recommendations. DH5α *Escherichia coli* cells were transformed with the ligation mixture and grown on 2YT plates in the presence of kanamycin. Plasmids were prepared from resistant cells and sequenced.

In situ hybridization on mouse tissues and chromosomal localization of human genes

In situ hybridization on whole mouse slices or isolated tissues was performed as described previously (Ruchon et al., 1998).

To determine the chromosomal localization of human NL-2 and NL-3 genes, a technique for mapping genes directly to banded human chromosomes was used. Metaphase chromosomes were obtained from lymphocytes cultured from normal human peripheral blood. Cells were synchronized with thymidine and treated with 5-bromodeoxyuridine (BrdU) during the last part of the S phase to produce R-banding. Biotin-labeling of the probe was done by nick-translation (Bionick, BRL) and the probe was visualized by indirect immunofluorescence.

Antibody production

To raise antibodies against the new peptidases, the cDNA sequences of each protein was compared to that of other members of the family and the sequence segment showing the less homology was used. These sequences are from amino acid residues 273 to 354 for NL-1, from 75 to 209 for NL-2 and from 143 to 465 for NL-3. These cDNA fragments were cloned in vector pGEX2T (Pharmacia Biotechnology) downstream from and in phase with Gluthatione-S-transferase (GST). Plasmids were transformed in *E. coli* strain AP401 and, induction of synthesis and purification of the fusion proteins were performed as recommended by the supplier. The NL polypeptides were cleaved from the fusion protein with thrombin and purified by SDS-PAGE. NL polypeptides were injected to rabbits or mice according to the following schedules: for rabbits, initial injection of 150 µg of protein with boosts of the same amount 4 weeks and 8 weeks following the initial injection; for mice, initial injection of 100 µg of protein followed by boosts of the same amounts 3 and 6 weeks later. A month after the last injection, sera were collected from the animals and tested by immunoblotting against the initial *E. coli*-produced antigens and the recombinant proteins produced in mammalian cell lines.

Production of monoclonal antibodies

cDNA fragments corresponding to amino acids segments of NLs selected to raise antibodies were used to construct a GST-fusion protein in E. coli. This fusion protein was purified from *E. coli* extracts by affinity chromatography on a glutathione-Sepharose column according to the supplier's instructions (Amersham-Pharmacia). After thrombin cleavage, the NL portion of the GST fusion protein was further purified by electroelution from a polyacrylamide gel. This material was used to immunise 4 mice (5 injections of ≈50 µg of NL polypeptide). Blood was collected from each mice after the immunisation schedule and the presence of antibodies in mice serum was assessed by ELISA using microtiter plates coated with NL polypeptide from E. coli extracts. Mice sera were also tested for the presence of NL antibodies by Western blotting extracts of mammalian cells transfected with the NL expression vectors. One mouse selected for its high titer of NL specific antibodies (as measured by ELISA) was sacrificed and its spleen cells were collected and immortalised by fusion with myeloma cells(strain: P3-X63Ag.653 from ATCC) as described previously (Crine 1985). Hybridoma cells were selected for their ability to grow in HAT selection medium and cloned by several rounds of limiting dilution. Hybridomas showing proper affinity and specificity to the enzymes NL-1, NL-2 and NL-3 where selected.

Expression of NLs in cultured mammalian cells and enzymatic assays

The cDNAs for NL-1 and NL-3 were cloned in vectors pcDNA3 or pRcCMV (Invitrogen) and introduced by transfection in mammalian cell lines according to procedures already described in our laboratory (Devault *et al.*, 1988a). Procedures to prepare extracts of cellular proteins or culture media were also described in previous papers (Devault *et al.*, 1988a; Lemay *et al.*, 1989). The presence of NLs in these extracts was monitored by immunoblotting using specific antibodies.

Extracts of cellular proteins and culture media were assayed for enzymatic activity. Two tests were performed. The first used [^{3}H]-Tyr-(D)Ala$_2$-Leu-enkephalin as substrate and was performed according to Lemay et al., (1989). The second used bradykinin as substrate and was performed as described by Raut et al. (1999).

RESULTS

Cloning of NL-1, a new member of the NEP family

The molecular cloning in the past few years of ECEs, PEX and KELL showed that all these proteins have between 50 and 60% similarity with NEP. This observation led us to believe that these peptidases are part of an extended family and that there could be still additional members to be discovered. To test this hypothesis, we aligned the amino acid sequences of the members of the NEP-like family and designed degenerate oligonucleotide primers to be used in RT-PCR reactions (Figure 1 and 2). These primers were located on either side of the HEXXH consensus sequence for zincins. Because they are highly degenerate, primers 1 and 2 were each subdivided into two pools, 1A-1B, and 2A-2B, respectively (Figure 2). Any PCR amplified DNA fragment that corresponds to a peptidase of the family should normally contain the consensus sequence and be easily recognized by sequencing of the cloned fragments. Using this strategy, we first performed PCR reactions with primer pairs 1A-3 and 1B-3. The amplified DNA migrates mostly as a smear starting at around 700 bp and going down to 100 bp. As the expected fragments should be around 550 bp, we isolated from the gel the section corresponding to DNA fragments longer than 500 bp. A second round of PCR reactions was performed with both crude PCR products of the first reaction and isolated DNA bands, using primers 2A-3 and 2B-3. The expected 296 bp fragment was seen on the gel (not shown).

Cloning of these DNA fragments generated approximately 350 clones, of which 44 were sequenced. Nine of these had no inserts or corresponded to sequences not related to the NEP family, 24 corresponded to NEP, 3 to PEX, and 8 corresponded to one putative new member of the family, since they all contained the HEXXH consensus sequence for zincins and showed 65% homology with mouse NEP (in boxes Figure 3). This fragment was then used to screen a mouse testis cDNA library, and allowed us to isolate a complete cDNA of 2592 nucleotides (Figure 3). The identity of this sequence with other members of the family is presented in Table I. This new member was called NL-1, for NEP-like peptidase 1.

Cloning of NL-2 and NL-3.

A strategy similar to that described for amplification of enzymes of the NEP family from mouse testis cDNAs was used with a human testis cDNA library using two different oligonucleotide primers. This time, DNA fragments of approximately 900 bp were obtained and cloned. Ten clones were sequenced, revealing the presence of NEP and two new peptidases of the family that we have called NL-2 and NL-3.

The NL-2 PCR fragment was 879 nucleotides in length and encoded a 293 amino acid residue segment probably located in the carboxy-terminal domain of this putative peptidase (in brackets Figure 4). This PCR fragment was then used to screen a lambda gt10 human brain cDNA library. It allowed the isolation of other cDNA fragments which overlap partially with the NL-2 PCR fragment. Fusion of these lambda clones and the PCR fragment resulted in an open reading frame of 770 amino acid residues. The use of 5' RACE protocols with human testis cDNA libraries allowed completion of the sequence of NL-2 ORF (Figure 4). This ORF codes for a putative protein that is about 80% identical to the mouse NL-1 protein (Figure 6). Across species, members of the NEP, PEX, ECEs sub-families have highly conserved sequences (more than 94% identity). Although a sequence identity of about 80% only exists between the novel human protein and mouse NL-1, these proteins share unique characteristics that make possible the fact that NL-2 protein may be the human homologue of NL-1. The identity of NL-2 with other members of the family is presented in Table I.

The 879 bp PCR fragment encoding NL-3 showed an open reading frame of 293 amino acid residues (Figure 5, in brackets). Sequence analysis of NL-3 showed that it was 94.2 % identical to an EST sequence from mouse embryonic tissue present in publicly accessible DNA data banks. This mouse EST sequence, commercially available from American Tissue and Cell Culture (ATCC), had been obtained previously by our laboratories.

Since Northern blot analysis of human tissues with the NL-3 PCR fragment showed the expression of this protein in spinal chord (see below), the same PCR DNA fragment was used to screen by hybridization a human spinal chord cDNA library constructed in phage λ vectors. One clone contained a full-length ORF of 752 amino acid residues that encompassed the 293 amino acid residue ORF of the PCR fragment. Further probing, cloning and sequencing lead to the obtention of NL-3 full sequence, shown in Figure 5.

Figure 6 presents a comparison of the amino acid sequence of the new NEP-like enzymes and Table I shows the extent of identity between members of the family.

Cellular distribution of NL-1, NL-2 and NL-3 peptidases

Determining the tissue distribution of NL-1, NL-2 and NL-3 may provide clues to identify the peptidergic systems in which they are involved. It will be particularly interesting to compare the tissue distribution of these peptidases with that of NEP and the ECEs to determine whether or not the physiological functions of NL-1 and/or NL-2 and/or NL-3 may overlap those of NEP and/or ECEs.

In situ hybridization (ISH), using our mouse cDNA, was used to determine the spatial and temporal expression of NL-1 during mouse development, as done previously for PEX (Ruchon et al., 1998)). Serial sections of whole foetal (12, 15 and 19 dpc) and adult mice (1, 3 and 6 days old) were hybridized with an [^{35}S]-labeled RNA probe. Figure 7 shows a section of mouse testis which was the only tissue identified to express NL-1 by this technique. Cells of seminiferous tubules are specifically labeled but spermatids located near the center of the tubule showed strongest labeling. These cells are in the last stage of maturation into spermatozoids. The presence of NL-1 in testis has now been confirmed by Northern analysis of mouse tissues (see Fig. 10). Other tissues express NL-1, when analyzed by RT-PCT, which is a more sensitive assay (not shown).

A similar approach was used to determine the localization of NL-3 using the mouse EST obtained from ATCC. Figure 8 shows sections of whole mouse at 17 days of embryonic development and 4 days post-natal. Several tissues are expressing this putative peptidase including brain, where it is associated with neurons (Figure 9), spinal chord, liver, spleen and bones. Labeling was stronger in bones from *Hyp* mouse, an animal model for hypophosphatemic rickets (Figure 8). In bones, NL-3 was found to be expressed by osteoblasts (not shown).

Northern blotting experiments were performed on several tissues with NL-2 and NL-3 probes. A Human Multiple Tissues Northern Blot (Clontech) was hybridized with specific probes. A single RNA band of approximately 4.0 kb was revealed by the probe for NL-2. Expression of NL-2 is restricted to brain and spinal cord (not shown). However, RT-PCR has shown the presence of this enzyme in testis (not shown).

A single RNA band of approximately 3.0 kb was detected with the specific probe for NL-3 (not shown). NL-3 expression was observed mainly in ovary, spinal cord and adrenal gland.

Chromosomal localisation of the human gene for NL-2 and NL-3

As a mean to get clues on the function of the new metallopeptidases in vertebrates, we have localized the new cDNAs on human chromosomes, in order to look for a possible link between the gene locus and mapped genetic diseases in humans. To do so, we have mapped the NL-2 and NL-3 genes by high-resolution fluorescence *in situ* hybridization (FISH). NL-2 was localized to chromosome band 1p36. Consistent with the cellular distribution of NL-2 in humans, genetic diseases of the CNS such as dyslexia, neural tube defect, neuroblastoma, neuronal type of Charcot-Marie-Tooth disease have all been mapped in this region and represent potential targets for a role of NL-2 in humans. NL-3 was localized to chromosome band

2q37. Consistent with a role of NL-3 in bones, a form of Albright hereditary osteodystrophy was mapped to the same chromosomal locus (Phelan et al., 1995).

In view of the foregoing, NL-2 and NL-3 are metallopeptidases that are assumed to be immediately useful as markers for a disease or disorder associated with human chromosomal locus 1p36 and 2q37, respectively. Their localization on a chromosome band associated with known diseases suggests that they may be expressed or co-expressed with one or more genes, as a cause or a consequence of disease development. It is possible that these enzymes are up or down regulated, alone or along with other genes involved in a disease. Therefore, antibodies or other ligands specific to NL-2 or NL-3 may be used for a diagnostic purpose, as well as primers or probes in diagnostic assays using nucleic acid hybridization or amplification techniques. Otherwise, primers or probes directed against the nucleic acids of NL-2 and NL-3 would be useful to map the mutations of a gene located in close proximity and involved in the disease. Therefore, no matter which exact function NL-2 and NL-3 gene products have, their chromosomic localization provides one diagnostic utility. This localization as well as the tissular distribution provide information as to the disease and tissue to be investigated to elucidate the exact function of these enzymes.

NL-1 resembles NL-2, sharing with the latter about 80% homology in the amino sequence and sharing structural characteristics such as the furin recognition sequence located at the proximal end of the ectodomain. NL-2 might be the human homologue of mouse NL-1. If such was the case, these two proteins would have a substantial degree of divergence and, maybe, different profiles of activity varying from one species to another.

Chromosomal localization of NL-1 was determined in mouse genome by Single Strand Conformational Polymorphism (SSCP) in collaboration with The Jackson Laboratory Backcross DNA Panel Mapping Resource. NL-1 was localized to the distal region of mouse chromosome 4 which corresponds to human chromosome region 1p36 where is located NL-2 gene. This reinforces our hypothesis that NL-1 and NL-2 are species variants.

Production of antibodies against NLs

Antisera collected from injected animals were first tested by immunoblotting on GST-antigen fusion proteins produced in *E. coli*. Antiserum from one rabbit recognized the NL-1-related polypeptide and antisera from one mouse and one rabbit reacted with the NL-3-related polypeptide (results not shown). The anti NL-1 antiserum and the mouse anti NL-3 antiserum, which appeared more specific than the rabbit antiserum, were next tested by immunoblotting on extracts of proteins and culture media from cells expressing NL-1 or NL-3 (see below).

Expression of NL-1 in CHO cells

The cDNA encoding the full-length NL-1 protein was cloned in the mammalian expression vector pcDNA3-RSV and transfected in CHO cells. Stable cell lines were established by selection with the drug G418 and tested by immunoblotting for the presence of NL-1.

Small amounts of NL-1 were found in the extracts of transfected CHO cells (results not shown). This intracellular species was sensitive to endo H digestion, indicating that the sugar moiety was not mature and suggesting ER localization (results not shown). The culture medium of transfected CHO cells showed the presence of soluble NL-1 (Figure 10). This extracellular species was resistant to endo H suggesting true transport through the late secretory pathway . The cDNA sequence of NL-1 predicts a type II transmembrane protein. The mechanism by which NL-1 is transformed into a soluble protein is not known presently. However, examination of the amino acid sequence revealed the presence of a putative furin cleavage site from residue 58 to 65 (Figure 3). A similar site is present in NL-2 sequence.

The soluble form of NL-1 was assayed for activity using [^{3}H]-Tyr-(D)Ala$_2$-Leu-enkephalin and bradykinin as substrates. Figure 11 shows that NL-1 can degrade the enkephalin substrate (K_m = 18±10 μM) and that this activity can be inhibited by phosphoramidon (IC_{50}=0.9±0.3 nM) and thiorphan (K_m=47±12nM), a general inhibitor of enzymes of the NEP family. Bradykinin is also a substrate for NL-1 (not shown).

Use of NL-1 amino-terminal domain to promote secretion

The observation that NL-1 ectodomain was secreted, possibly through cleavage of the transmembrane segment by furin, raised the possibility to promote secretion of exogenous proteins that could be spliced to NL-1 amino-terminal domain (from initiator methionine to the furin site). To test this hypothesis, the ectodomain of NL-3 (from the third cysteine to the end) was spliced to NL-1 amino-terminal domain using a PCR strategy and the recombinant DNA cloned in expression vector pRcCMV. The fusion protein was expressed by transfection of the vector in COS-1 and HEK 293 cells. The culture media of transfected cells was analyzed by immunoblotting using the mouse antiserum against NL-3. Figure 12 shows the presence of NL-3 in the spent culture media of both COS-1 and HEK 293 cells. This result shows that NL-1 amino-terminal domain can be used to promote secretion of exogenous proteins.

The soluble form of NL-3 was assayed for activity using [^{3}H]-Tyr-(D)Ala$_2$-Leu-enkephalin as substrate. No activity was found.

The previous experiment showed that it was possible to use the amino-terminal domain of NL-1 to promote secretion of an otherwise membrane attached protein ectodomain. To verify whether the same strategy could be used to promote secretion of small peptides, a PCR strategy was used to splice human β-endorphin to the amino-terminal domain of NL-1 and the recombinant DNA was cloned in vector pRcCMV. The fusion protein was expressed by transfection of the vector in COS-1 and HEK 293 cells. The culture media of transfected cells was collected 48h after transfection and the peptides purified as described previously (Noël et al., 1989). The presence of β-endorphin in the extracts was detected by radioimmunoassay. The results showed that both COS-1 and HEK 293 cells produced approximately 100 pg of β-endorphin per ml of culture medium. Therefore, the N-terminus of LN-1 and NL-2 which ends with a furin-recognition site will be useful to produce the soluble form of a protein of interest.

SECTION 2)

MATERIALS AND METHODS

DNA manipulations

All DNA manipulations, phage library screening, and plasmid preparations were performed according to standard protocols (Ausubel 1988; Sambrook 1989). Site-directed mutagenesis was performed using a PCR-based strategy as described previously (Le Moual 1994).

mRNA purification and RT-PCR protocol for identification of new members of the neprilysin family

mRNAs were prepared from mouse testis using Quick Prep Micro mRNA purification kit (Pharmacia Biotech). First strand cDNA was synthesized from 1 µg of mRNA using the First-Strand cDNA synthesis kit (Pharmacia Biotech).

Two sense primers, oligonucleotide 3817 (5'-TGGATGGAT/CGA/CIGG/AIACIA/CA-3') and oligonucleotide 3719 (5'-A/GTIGTITTT/CCCIGCIGGIA/GT/AIC/TTG/CCA-3') corresponding respectively to amino acid residues 459 to 465 and 552 to 560 of NEP sequence, and one antisense primer, oligonucleotide 3720 (5'-AIICCICCIA/TC/TA/GTCIGCIG/AC/TA/GTTT/CTC-3') corresponding to amino acid residues 646 to 654 (see Fig. 1 and 2), were synthesized. PCR was performed with 5 µl of cDNA template and 1 µl of Taq DNA polymerase in a final volume of 100 µl, containing 1 mM $MgCl_2$, 2 µM of each oligonucleotide 3817 and 3720, 200 µM of each dNTP and 5% DMSO. Cycling profiles included an initial denaturation step of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 40°C and 1.5 min at 72°C, and a final extension step at 72°C for 10 min. One half of the amplified DNA was fractionated on a 2% agarose gel and fragments ranging in size between 500-700 bp were purified and resuspended in a final volume of 50 µl. A second round of PCR was done with primers 3719 and 3720, using as template either 10 µl of the first PCR reaction or 5 µl of the purified fragments, and the new PCR products were ligated in pCR2.1 vector (Invitrogen). Several identical clones corresponded to a potential new member of the NEP family. We called this member NL1 for NEP-like 1.

Cloning of full-length NL1 cDNA

The cloned NL1 PCR fragment was used as probe to screen a mouse testis λ Uni-ZAP^{TM}XR cDNA library (Stratagene). Twelve out of a hundred positive phages were plaque purified and subcloned into pBS SK vector (Stratagene). As the longest clone analyzed presented an incomplete ORF (pBS-NL1A), 5'RACE with primers located in vector (5'-TAGTGGATCCCCCGGGCTGCAG-3', sense primer) and NL1 (5'-ACCAAACCTTTCCTGTAGCTCC-3', antisense primer, nt 1303 to 1324 of NL1; was subsequently performed on the DNA of the remaining semi-purified positive clones. Amplification was performed with 1µl of Vent polymerase in a final volume of 100 µl containing 50 ng of DNA, 4 mM of $MgSO_4$, 1µM of each oligonucleotide, 200 µM of each dNTP and 10% DMSO. Cycling parameters included an initial denaturation step of 1 min at 94°C, 25 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C, and an incubation of 10 min at 72°C. A PCR fragment of the expected length was subcloned into pCR2.1 vector (clone pCR-NL1A), but sequencing revealed no initiator ATG codon. A nested 5'RACE was then performed on mouse testis cDNA using the Marathon Ready cDNA kit (Clontech) with sense oligonucleotides AP1 and AP2 (from the kit) and NL1 antisense oligonucleotides 5'-CCTGAGGGCTCGTTTTACAACCGTCCT-3' (nt 503 to 529 of NL1) and 5'-CTCATCCCAGGAGAAGTGTAGCAGGCT-3' (nt 475 to 502 of NL1) as recommended by the supplier. The resulting fragment was cloned into pCR2.1 vector (pCR-NL1B). Since only ten bp were missing for the initiator ATG codon, we reconstructed the 5' end of the cDNA by PCR-amplifying clone pCR-NL1A with sense primer 5'-CCACCATGGTGGAGAGAGCAGGCTGGTGTCGGAAGAAG-3' (nt 332 to 364 of NL1; the 10 missing nucleotides are underlined) and antisense primer 5'-ACCAAACCTTTCCTGTAGCTCC-3' (nt 1303 to 1324 of NL1) using Vent polymerase as described above. The DNA fragment was then inserted into pCR2.1 (clone pCR-NL1C). The entire ORF was reconstituted following digestion of pBS-NL1A and pCR-NL1C with *Eco*RI and *Pfl*MI. The 5' end of NL1 cDNA was excised from pCR-NL1C and ligated into pBS-NL1A at the corresponding sites, resulting in plasmid pBS-NL1B.

For expression studies, a *Bam*HI/*Apa*I fragment generated out of pBS-NL1B, corresponding to the full length cDNA of NL1, was inserted into pCDNA3/RSV [18] vector.

Production of polyclonal antibodies

A plasmid for the production in *Escherichia coli* of a GST fusion protein with NL1 was constructed using pGEX-4T-3 expression vector (Pharmacia Biotechnologies). A 255 bp fragment from NL1 was amplified by PCR with Vent polymerase using sense primer 5'-GCTACGGGATCCGTGGCCACTATGCTTAGGAA-3' (nt 1139 to 1158) and antisense primer 5'-CGATTGCTCGAGTGGGAACAGCTCGACTTCCA-3' (nt 1377 to 1396). Both pGEX-4T-3 and the PCR product were digested with *Bam*HI and *Xho*I and ligated. The recombinant protein was produced and purified according to the supplier's instructions. Five weeks old female balb/c mice were immunized at monthly intervals for 3 months with 20 µg of the recombinant NL1 fragment in Freund's adjuvant and antisera were subsequently collected.

Cell culture and transfection

Human Embryonic Kidney (HEK 293) cells were maintained in DMEM medium containing 10% fetal bovine serum (FBS), and supplemented with penicillin at 60 µg/ml, streptomycin at 100 µg/ml and fongizone at 0,25 µg/ml. Transfections of cells with appropriate plasmids were performed by the calcium/phosphate-DNA co-precipitation method (Chang 1987). To establish permanent cell lines, G418 selection was initiated 48 h after the transfections at 400 µg/ml for 12 days and gradually decreased at 100 µg/ml.

LLC-PK$_1$ cells transfected with pRcCMV-sNEP were maintained as described previously (Lanctöt 1995).

Immunoblot analysis

For immunoblot analysis, cells were incubated for 16 h in synthetic DMEM medium containing 2mM sodium butyrate. Cellular proteins were solubilized as previously described (Dion 1995). Secreted proteins recovered in culture media were concentrated approximately 10 fold by ultrafiltration. Immunoblot analysis were performed using the NEN Renaissance kit with the polyclonal antibody specific to NL1 or the α1-antitrypsin inhibitor antibody (Calbiochem; LaJolla, CA) followed by the appropriate horseradish peroxidase-conjugated IgG (Vector Laboratories).

For the glycosylation studies, proteins were incubated with endoglycosidase H (endoH) or peptide:N-glycosidase (PNGaseF) as suggested by the distributor (NEB).

Enzymatic activity assays

NL1 activity was monitored and compared to sNEP activity using (Tyrosyl-[3,5-^{3}H])(D-Ala$_2$)-Leu$_5$-enkephalin (50 Ci/mmol) (Research Products International Inc.), as already described (Dion 1995; Devault 1988). K_m values were determined by the isotope-dilution method. The inhibitory effects of phosphoramidon and thiorphan were also assessed as previously described (Dion 1995).

HPLC analysis of the hydrolysis of Leu-enkephalin

Five µg of Leu$_5$-enkephalin were incubated at 37°C for one hour in 50mM MES, pH 6.5, with concentrated culture medium of HEK 293 cells expressing NL1 (~300 µg of total proteins) or LLC-PK$_1$ cells expressing sNEP (~30 µg of total proteins), in absence or presence of 0.1 mM phosphoramidon. Hydrolysis products were separated by reversed-phase HPLC as described previously [23]. Tyr-Gly-Gly and Phe-Leu were both identified by elution profiles of synthetic marker peptides.

Northern blot analysis

A mouse multiple tissue poly(A)$^+$ mRNA blot (Clontech) was hybridized with a [^{32}P]dCTP random primer labelled probe in ExpressHyb solution (Clontech). The blot was washed according to the manufacturer's recommendations and exposed to Fuji RX film for 7 days at -80°C with intensifying screens.

RT-PCR screening of mouse tissues

First strand cDNA synthesis was performed with 1 μg of total RNA from mouse tissues and oligo(dT) as primer, using Gene Amp RNA PCR Core Kit (Perkin Elmer). For the PCR reactions, primers 5'-TGGCGAGAGTGTGTCAGCTATGTC-3' and 5'-CTTCCAAAATGTAGTCAGGGTAGCCAATC-3' were used with Taq polymerase. One tenth of the PCR products were visualized on a 4% agarose gel.

In situ hybridization

To construct a plasmid for the synthesis of cRNA probes for ISH, pCR-NL1A was used as template to amplify a 452 bp fragment by PCR with sense primer 5'-GGAGCCATAGTGACTCTGGGTGTC-3' (nt 416 to 439) and antisense primer 5'-GACGCTCAGCAGGGGCTCAGAGTC-3' (nt 842 to 865). The amplification product was inserted into pCRII vector (Invitrogen). Synthesis of riboprobes and protocols for ISH were as described previously (Ruchon 1998).

RESULTS

Cloning and sequence analysis of mouse NL1 cDNA

In order to isolate cDNAs for new members of the NEP family, we developed an RT-PCR strategy based on fact that NEP, ECE-1 and PHEX share regions of significant sequence identity. Following RT-PCR on testis mRNAs with nested primers, a DNA fragment of approximately 300 bp was amplified. This DNA fragment was cloned and the plasmids from 24 independent colonies were sequenced: 3 clones had no insert, 4 clones had DNA fragments not related to the NEP family, 7 clones had sequences corresponding to mouse NEP and 3 clones had sequences corresponding to mouse PHEX, showing that our approach efficiently allowed the identification of members of the family. Moreover, 7 identical clones had a new cDNA presenting sequence similarities to members of the NEP family. The full-length cDNA was subsequently obtained by screening a mouse testis λ cDNA library followed by 5'RACE, as described under *Materials and Methods*. Its nucleotide and deduced amino acid sequences confirm that we cloned a novel NEP-like protein, referred to thereafter as NL1.

NL1 cDNA spans 2925 nt, including a 5'-untranslated region of 331 nt, an open reading frame of 2295 nt from nt 332 to nt 2626, and a 3'-untranslated region of 299 nt. The sequence surrounding the proposed initiator ATG conforms to the Kozak consensus (Kozak 1986). The deduced amino acid sequence of NL1 reveals a putative type II transmembrane protein of 765 amino acid residues encompassing a short N-terminal cytoplasmic tail, a unique putative transmembrane domain, and a large C-terminal extracellular domain. The ectodomain contains nine potential N-glycosylation sites (Asn-X-Ser/Thr) and ten cysteine residues corresponding to those conserved among all the members of the family, which are presumably involved in proper folding and in maintenance of the protein in an active conformation. All amino acid residues known to be part of the active site of NEP are present in NL1. The predicted protein presents greater similarities to NEP than to any other member of the family.

Although NL1 shares numerous features with proteins of the neprilysin family, a notable aspect distinguishes it from the others : the first conserved cysteine residue of the ectodomain is more distant (34 amino acid residues) from the predicted transmembrane domain in NL1 than it is in NEP (9 residues) or any other members of the family. Moreover, we noticed a putative furin cleavage site (-Arg_{58}-Thr-Val-Val-Lys-Arg_{63}-) between the end of the transmembrane domain and the first cysteine. This observation suggests that NL1 could exist as a secreted rather than a membrane-bound protein.

NL1 expression in HEK 293 cells

HEK 293 cells were transfected with pCDNA3/RSV expression vector containing NL1 cDNA, and a permanent cell line was established as described under *Materials and Methods* (HEK/NL1 cells) . Immunoblotting with a polyclonal antibody showed that after 16h of culture, most NL1 was present in the culture medium with small amounts of the enzyme in the cell extract. Secreted and cell-associated NL1 had apparent molecular masses of approximately 125 and 110 kDa, respectively. To characterize the glycosylation state of NL1, we next submitted the recombinant protein to deglycosylation by peptide : N-glycosidase F (PNGase F) and endoglycosidase H (endo H). PNGase F removes high mannose as well as most complex N-linked oligosaccharides added in the Golgi complex. In contrast, endo H removes N-linked oligosaccharide side chains of the high mannose type found on proteins in the RER but which have not yet transited through the Golgi complex; thus, resistance to endo H can be used as an indication that the protein has traveled through the Golgi complex. PNGase F treatment showed that the cell-associated and secreted NL1 were N-glycosylated as their electrophoretic mobility increased following digestion. However, the secreted NL1 migrated as a doublet after PNGase F treatment, with one band co-migrating with cell-associated form and the second having a slower rate of migration. Since untreated and endo H-digested secreted NL1 are seen as single bands by SDS-PAGE, our observation suggests that a proportion of secreted NL1 undergoes further post-RER postranslational modification that renders some of the N-linked oligosaccharides resistant to PNGase F digestion.

In contrast to secreted NL1, NL1 from cell extract was sensitive to endo H treatment. This result shows differences in the glycosylation state of the two species and suggests that the cell-associated form observed in transfected cells is an intracellular species that has not traveled through the Golgi complex.

Processing of NL1 by a subtilisin-like convertase

To determine whether a member of the mammalian subtilisine-like convertase family is responsible for NL1 presence in the culture medium of transfected cells, we co-transfected transiently HEK 293 cells with a constant amount of plasmid pCDNA3/RSV/NL1 and increasing amounts of plasmid pCDNA3/CMV/PDX (Benjannet 1997). This latter vector promotes the expression of the α1-antitrypsin Portland variant, α1-PDX, a known inhibitor of subtilisin-like convertases (Anderson 1993). Immunoblot analysis of the culture media of cells expressing both NL1 and α1-PDX indicated that NL1 secretion was strongly inhibited by the presence of α1-PDX: a relation was observed between the amounts of α1-PDX and the level of inhibition of NL1 secretion.

To confirm that proteolysis by the subtilisin-like convertase occurred at the putative furin cleavage site identified in NL1 ectodomain (-Arg_{58}-Thr-Val-Val-Lys-Arg_{63}-), the amino acid residues Asn_{62}-Gly_{63} were substituted for Lys_{62}-Arg_{63} by site-directed mutagenesis in vector pCDNA3/RSV/NL1 and the mutated vector used to establish HEK 293 cells expressing the mutant protein (HEK/NL1mut cells). Immunoblot analysis of the culture media of HEK/NL1mut cells showed that the mutation totally abolished secretion of NL1. Furthermore, an additional form of NL1 with a molecular mass of 127 kDa was detected in the extract of these cells. This new species was resistant to endo H digestion and was found associated with membranes when HEK/NL1mut cells were fractionated according to Chidiac *et al.* 1996 (result not shown).

NL1 enzymatic activity

Culture media from HEK 293 and HEK/NL1 cells were tested for enzymatic activity using as substrate (Tyrosyl-[3,5-^{3}H])(D-Ala_2)-Leu_5-enkephalin, a known NEP substrate. Activity was detected in the culture medium of HEK/NL1 cells but not in that of HEK 293 cells. This activity increased linearly with the amounts of NL1 and with the incubation period, indicating that degradation of the substrate was due to NL1 enzymatic activity.

We next characterized NL1 enzymatic parameters using the same substrate and compared them to those of an engineered soluble form of NEP (sNEP) (Lemay 1989). NL1 affinity for D-Ala$_2$-Leu$_5$-enkephalin was slightly higher than that of sNEP as shown by their K_m values of 18 μM and 73 μM, respectively. Inhibition assays showed that phosphoramidon had similar effects on NL1 and sNEP activity, with IC$_{50}$ values of 0.9 nM and 0.5 nM respectively, and that thiorphan, a specific inhibitor of NEP, inhibited NL1 with an IC$_{50}$ of 47 nM, as compared with an IC$_{50}$ of 8 nM for NEP.

Very low levels of phosphoramidon-sensitive activity was detected in extracts of HEK/NL1 cells (data not shown) consistent with the small amounts of NL1 observed by immunoblotting.

To determine whether NL1 had cleavage site specificity similar to NEP, we incubated Leu$_5$-enkephalin in the presence of NL1 recovered from the medium of HEK/NL1 cells or in the presence of sNEP, and analyzed the degradation products by RP-HPLC. Peaks co-migrating with standard Tyr-Gly-Gly and Phe-Leu peptides were observed in both RP-HPLC profiles, indicating that both enzymes cleaved the substrate at the Gly$_3$-Phe$_4$ peptide bond. This enkephalin-degrading activity was completely inhibited by phosphoramidon (1 μM).

Tissue and cellular distribution of NL1 mRNA

Tissue distribution of NL1 mRNA was determined by Northern blot analysis with a specific probe corresponding to the 5'end of the coding region of NL1 cDNA. A single transcript of 3.4 kb was detected exclusively in testis among all the mouse tissues tested. Mouse tissues were also screened by RT-PCR. Using this more sensitive technique, expression of NL1 was observed in several other tissues including heart, brain, spleen, lungs, liver and kidney. Consistent with the Northern blot results, RT-PCR analysis, although not strictly quantitative, detected more NL1 mRNA in testis than in other tissues.

To gain more insight into NL1 mRNA distribution, we examined by ISH cryostat sagital sections from a 4-day newborn mouse, as well as sections from a 16-day old animal (p16) and adult tissues (heart, brain, spleen, lungs, liver, kidney and testis). The presence of NL1 mRNA was detected only in adult testis. Only the germinal cells in the luminal face of the seminiferous tubules were labeled. These cells were identified as round and elongated spermatids in all spermiogenesis maturational stages. Neither spermatozoa nor spermatocytes, spermatogonies or Sertoli cells were labeled. Interstitial cells were also negative. Controls were performed with sense riboprobes, which produced only nonspecific background (data not shown). The 4-day old mouse sagital sections and all other tissues tested were negative.

DISCUSSION

The great interest in members of the Neprilysin family as putative therapeutic targets, and the recent discovery of new members of this important family of peptidases led us to investigate whether additional members of the family remained to be identified. Using a PCR-based strategy, we cloned, from mouse testis, a partial cDNA encoding a new NEP-like enzyme that we called NL1. Analysis of the amino acid sequence encoded by the full-length NL1 cDNA revealed that this member of the family resembles NEP the most: 55% identity and 74% similarity. Recently, the primary structure of a new zinc metallopeptidase from total mouse embryo was reported (Ikeda 1999). This enzyme, called SEP, is found either as a soluble or a cell-associated form due to alternative splicing. NL1 shows only 3 amino acid differences with the soluble form of SEP indicating that secreted SEP and NL1 are the same enzyme. Our cloning strategy did not allow characterization of the cell-associated form of NL1 which is a minor species in mouse testis (Ikeda 1999).

The amino acid sequence of NL1 predicts a topology of a type II integral membrane glycoprotein that is similar to the other members of the family. Treatment of the recombinant protein with PNGase F showed that indeed NL1 possesses N-linked carbohydrate side chains. However, it is not possible to determine precisely whether all nine putative N-glycosylation sites are used, but the 30 kDa decrease in molecular mass upon PNGase F treatment suggests that most are glycosylated. It has already been shown that all asparagine residues in a Asn-X-Ser/Thr consensus are glycosylated in rabbit NEP expressed in COS-1 cells and that sugar moieties increase the stability and enzymatic activity of the protein and facilitate its intracellular transport (Lafrance 1994). Three of NEP glycosylated Asn residues (Asn 145, Asn 285 and Asn 628) are conserved in NL1 (Asn 163, Asn 303 and Asn 643). Amongst these residues, Asn 145 and Asn 628 have been reported to influence NEP enzymatic activity (Lafrance 1994). In the same work, it has also been shown that the effect of sugar addition on folding and intracellular transport of NEP is a cumulative effect of all glycosylation sites rather than a contribution of any particular one. Glycosylation of NL1 may share similarities with that of NEP since we found their primary structures and enzymatic activities to be very similar.

Surprisingly, expression of the cDNA by transfection of HEK 293 cells showed that most of the enzyme was secreted in the culture medium. The small amount of NL1 associated with the cells was endo H-sensitive, suggesting that the cell-associated enzyme is a species that has not yet left the RER. The presence of a furin cleavage site in NL1 sequence between the predicted transmembrane domain and the first conserved cysteine residue of the ectodomain led us to believe that a member of the mammalian subtilisin-like family of convertases was responsible for the presence of NL1 in the culture medium. These enzymes are involved in processing a variety of precursor proteins such as growth factors and hormones, receptors, plasma proteins, matrix metalloproteinases, metalloproteases-desintegrins and viral envelope glycoproteins [for a review see: (Nakayama 1997). Site-directed mutagenesis of the furin cleavage site (-Arg_{58}-Thr-Val-Val-Lys-Arg_{63}-) and expression of α1-PDX, a potent inhibitor of mammalian subtilisin-like convertases (Anderson 1993), confirmed that a member of this family of endoproteases was involved in NL1 secretion presumably by cleaving in carboxy-terminus of Arg_{63}. There are only a few examples of proteins which are processed from a membrane-bound precursor to a secreted form following cleavage by subtilisin-like convertases; these include meprin and collagen XVII (Milhiet 1995; Schacke 1998). Three members of the subtilisin-like family of convertases, namely furin, PC4 and PC7, are known to be expressed in germ cells (Nakayama 1992; Torri 1993; Seidah 1992, 1996). Whether one of these convertases generates secreted NL1 from its membrane form is under current investigation. In any case, NL1 is the only known member of the neprilysin family that is secreted. This unique feature suggests that NL1 plays its physiological role in a context different from that of the membrane-bound peptidases, thereby diversifying the role of the peptidases of the neprilysin family. It is of interest that circulating forms of NEP in blood and urine have been described, but they have generally been related to pathological or stressful conditions (Almenoff 1984; Deschodt-Lanckmann 1989; Johnson 1985; Soleilhac 1996; Aviv 1995).

We have observed in cells expressing NL1 mutated at the furin cleavage site the appearance of a species resistant to digestion by endo H. This mutated protein was associated with cellular membranes. Taken together, these results indicate that NL1 is first synthesized and inserted in the RER membrane as a type II transmembrane protein. During intracellular transport, NL1 is converted to a soluble form by the action of a member of the mammalian subtilisin-like convertases. The identity of the cellular compartment where this process occurs is not known. However, mammalian subtilisin-like convertases are usually active in post-Golgi compartments of the secretory pathway suggesting that processing of NL1 from the membrane bound form to the soluble form is a post-Golgi event.

Despite almost total abrogation of NL1 secretion, we observed only a slight accumulation of endo H-resistant NL1 in cells either co-expressing α1-PDX and NL1 (result not shown) or expressing mutated NL1. This observation suggests that unprocessed NL1 is rapidly degraded. A similar behavior was reported for the Notch1 receptor expressed in the furin-deficient cell line LoVo (Logeat 1998). The mechanism(s) by which these unprocessed proteins are degraded is still unknown. It is interesting to point out that the spliceoform of SEP that has lost a 23 amino acid peptide, including the furin cleavage site, generates a cell-associated endo H-sensitive molecule (Ikeda 1999).

The most important observation regarding the NL1 primary structure is the conservation of residues which in NEP are essential for catalysis and binding of substrates or inhibitors. This finding suggests that NL1 could effectively act as an endopeptidase with a catalytic mechanism similar to that of NEP. This hypothesis was supported by the demonstration that D-Ala_2-Leu_5-enkephalin, a peptide substrate often used to monitor NEP activity, was also an excellent NL1 substrate. The affinity of NL1 for D-Ala_2-Leu_5-enkephalin was even higher than that of NEP, as reflected by a K_m value 4- to 5-fold lower. Furthermore, two well known NEP inhibitors, phosphoramidon and thiorphan, also abolished NL1 activity. Phosphoramidon, which inhibits NEP as well as ECE-1 activity, albeit to a lesser extent (Turner 1996), had very similar effects on NL1 and NEP, with an IC_{50} value for NL1 varying not more than two-fold from the value determined for NEP. Thiorphan, considered to be a more specific inhibitor of NEP, also inhibited NL1 activity, with an IC_{50} six-fold greater than that for NEP. These results suggest that the active sites of NL1 and NEP are similar. This hypothesis is supported by the observation that secreted SEP degraded a set of peptides known to be NEP substrates, including substance P, bradykinin and atrial natiuretic peptide (Ikeda 1999). Taken together, these results illustrate the importance of identifying and characterizing other member of the family for the design of highly specific inhibitors.

In agreement with the enzymatic parameters demonstrating that NL1 and NEP have similar catalytic sites, we have observed that both enzymes cleaved Leu_5-enkephalin at the same peptide bond. This result suggests that NL1 hydrolyzes peptide bonds on the amino side of hydrophobic amino acid residues as does NEP (Turner 1985). However, several other peptides will have to be tested to confirm this specificity and to determine whether NL1 has dipeptidyl carboxypeptidase activity as was shown for NEP (Malfroy 1982; Bateman 1989; Beaumont 1991) and more recently for ECE-1 (Johnson 1999).

RT-PCR experiments with specific primers for the soluble and cell-associated forms of SEP showed a wide tissue distribution of the enzyme with the soluble form of SEP being predominant in testis and the cell-associated form in other tissues (Ikeda 1999). Our RT-PCR results confirmed the wide tissue distribution of NL1. However, Northern blotting and *in situ* hybridization experiments indicated that expression of NL1 is predominant in germ cells of mature testis. Interestingly, proenkephalin mRNA has been shown to be expressed in germ cells and somatic cells of the testis (Torii 1993, Seidah 1992; Kew 1989; Mehta 1994; Kilpatrick 1986, 1987). Specific functions for testicular enkephalin peptides have not yet been defined, but it is believed that they could act as intratesticular paracrine/autocrine factors. In addition to their putative role as mediators of testicular cell communication, it has also been demonstrated that proenkephalin products synthesized by spermatogenic cells during spermatogenesis are stored in the acrosome of human, hamster, rat and sheep spermatozoa and are release from sperm following acrosomal reaction (Kew 1990). It has thus been proposed that proenkephalin products may act as sperm acrosomal factors during the fertilization process as well as intratesticular regulators secreted by spermatogenic cells. Since Leu$_5$-enkephalin was found to be a good substrate for NL1, opioid peptides originating from proenkephalin could serve as physiological substrate for this new enzyme. In this way, NL1 would serve to regulate the activity of these bioactive peptides.

Testis is the only tissue where the soluble form of SEP is predominant (Ikeda, 1999), suggesting a testis-specific alternative splicing. Expression of testis-specific molecular species of peptidases or prohormones, arising through diverse mechanisms, has been documented in the past (Howard 1990; Jeannotte 1987). However, the physiological significance of these testis-specific species is not always clear. In the case of NL1 or SEP, it might allow local constitutive secretion by germinal cells of an otherwise cell-associated enzyme, to regulate spermatogenesis much like several other proteolytic enzymes of the seminiferous tubules (Monsees 1998). Alternatively, it might allow accumulation in acrosome with proenkephalin peptides and release upon acrosomal reaction. More exhaustive studies concerning NL1 localization and physiological substrate identification will be needed to understand its role in the testis and possibly in the fertilization process.

Cloning of other members of the family

To find other members of the NEP-like family, we will use the same RT-PCR strategy to amplify mRNA isolated from tissues known to be regulated by peptidergic systems (brain, thymus, kidney, heart, lung, ovary, pancreas, bone, bone marrow and lymphoid cells). In fact, many of these tissues are known to express at least one member of the family and/or to control a peptidergic pathway on which peptidase inhibitors have major effects. Amplified fragments will be cloned and the resulting clones will be sequenced and compared to the sequence of known peptidases, as described above. Pairs of degenerate primers in other highly conserved regions will also be designed to increase the possibility of cloning other relevant peptidases.

DISCUSSION

As discussed above, peptidases of the NEP family known to date have often been found to play important physiological roles. This is certainly true for NEP itself, ECEs and PEX, (see review above). For this reason, some of these enzymes (as it was the case for NEP and ECE in the past) might be interesting targets for the design of inhibitors that in turn could be used as therapeutic agents in various pathological conditions. However, it is of some concern that inhibitors designed for one enzyme may also inhibit to some extent other members of the family. This lack of specificity for an inhibitor used as a therapeutic agent in the long term treatments such as those used as antihypertensive agents for instance, may cause unforeseen problems due to unwanted side effects. The objectives of the present work was to develop a strategy to clone new members of the NEP family of peptidases. The results presented in this report clearly show that our strategy can be successful. We have determined the complete or partial nucleotide sequence of three cDNAs encoding putative enzymes of the NEP family.

These cDNA sequences are valuable tools and may be used to:

Produce antibodies

As shown in the present work, knowledge of NL cDNA sequences can be used to raise specific antibodies. For example but not exclusively, regions of less homology between the peptidases (amino acid residues 50 to 450) can be used to synthesize peptides whose sequences are deduced from the translation of the cDNAs, and/or bacterially-expressed fragments of the cDNAs fused for example but not exclusively to GST may be purified and injected into rabbits or mice for polyclonal or monoclonal antibody production. These antibodies can be used to:

- identify by immunohistochemistry the peptidergic pathways in which the peptidases are functioning;
- study the physiopathology of NL-enzymes by immunoblotting or immunohistochemistry on samples of biological fluids or biopsies;
- set up high through put screening assays to identify NL-enzymes inhibitors. This can be done for example but not exclusively by using the antibodies to attach the NL-enzymes to a solid support;
- purify NL-enzymes with said antibodies by immunoprecipitation or affinity chromatography by identifying antibodies capable of selectively binding to the NL-enzymes in one set of conditions and releasing it in another set of conditions typically involving a large pH or salt concentration change without denaturing the NL-enzyme;
- identify antibodies that block NL-enzymes activities and use them as therapeutic agents. Blocking antibodies can be identified by adding antisera or ascite fluid to an *in vitro* enzymatic assay and looking for inhibition of NL-enzymes activities. Blocking antibodies could then be injected to normal or disease model animals to test for *in vivo* effects.

Derive specific RNA or DNA probes

As shown in the present work, knowledge of the nucleotide sequence of the members of the NEP-family allows nucleotide sequence comparisons and facilitate the design of specific RNA or DNA probes by methods such as but not exclusively molecular cloning, *in vitro* transcription, PCR or DNA synthesis. The probes thus obtained can be used to:

- derive specific probes or oligonucleotides for RNA and DNA analysis, such as Northern blot and *in situ* hybridization, chromosome mapping or PCR testing. These probes could be used for genetic testing of normal or pathological samples of biological fluids or biopsies;

- make vectors for gene knock-out or knock-in in mice. The long range PCR technique and/or screening of a mouse genomic library with probes derived from the 5'-end of the cDNAs can be used to isolate large exon/intron regions. We will then substitute one or more of the cloned genomic DNA exons for the neomycin resistance expression cassette for producing homologous recombination and knock-out mice. Alternatively, cDNAs coding for NLs will be used to overexpressed each of these enzymes in transgenic mice. The cDNAs will be cloned downstream from a promoter sequence, and injected in fertilised mouse eggs. Depending on specific questions to be answered, the chosen promoter sequence will allow expression of the peptidases either in every tissues or in a cell- or tissue-specific manner. Injected eggs will be transferred into foster mothers and the resulting mice analysed for peptidase expression;
- replace defective NL genes in a gene therapy strategy. The NL full length cDNAs could be cloned under the control of a constitutive or inducible promoter having a narrow or wide range of tissue expression and introduced with appropriate vectors in subjects having defective genes;
- synthesise oligonucleotides that could be used to interfere with the expression of the NLs. For example but not exclusively, oligonucleotides with antisens or ribozyme activity could be developed. These oligonucleotides could be introduced in subjects as described above;
- isolate other members of the family. Screening cDNA and/or genomic libraries with these cDNA probes at low stringency may allow to clone new members of the NEP-like family. Alternatively, alignment of the sequences may allow one to design specific degenerate oligonucleotide primers for RT-PCR screening with mRNA from tissues such as but not exclusively, the hearth and the brain.

Production of recombinant NL-enzymes

As shown in the present work, recombinant active NL-enzymes can be obtained by expression of NL-cDNAs in mammalian cells. From past experience with neprilysin, another member of the family (Devault *et al.*, 1988; Fossiez *et al.*, 1992; Ellefsen *et al.*, submitted), expression can also be performed in other expression systems after cloning of NL-cDNAs in appropriate expression vectors. These expression systems may include but not exclusively the baculovirus/insect cells or larvae system and the *Pichia* *pastoris*-based yeast system. Production of recombinant NL-enzymes includes the production of naturally occurring membrane bound or soluble forms of the proteins or genetically engineered soluble forms of the enzymes. The latter can be obtained by substituting the cytosolic and trans-membrane domain by a cleavable signal peptide such as that of proopiomelanocortin, but not exclusively, as done previously (Lemay *et al.*, 1989) or by transforming by genetic manipulations the non-cleavable signal peptide membrane anchor domain into a cleavable signal peptide, as done previously (Lemire *et al.*, 1997) or by fusion of the ectodomain of NL-enzymes to the amino-terminal domain (from the initiator methionine to amino acid residue 300) of naturally occurring soluble NLs such as, but not exclusively, NL-1 as done in this work.

These recombinant NLs could be used to:

- find a substrate. A substrate can be identified using one of the following.
  - Screening of existing bioactive peptides. Peptides are incubated in the presence of NL-enzymes and subsequently analysed by HPLC for degradation. Degradation is observed by disappearance of the peak of substrate and the appearance of peaks of products;
  - Screening phage libraries specifically designed for the purpose (phage display library). Each phage expresses at its surface, as part of its coat protein, a random peptide sequence preceded by a peptide sequence recognisable by an antibody or any other sequence-recognizing agent. This latter sequence serves to attach the phage to a solid support. Upon addition of the NL-enzyme the random sequences that are NL substrate are cleaved, releasing the phage. After several rounds of cleavage, the phage sequence is determined to identify the peptide segment recognized by the enzyme.
  - Extract of the tissue where the enzyme is expressed is collected and prepared for chromatographic analysis (HPLC, capillary electrophoresis or any other high resolution separation system) by denaturing the extracted proteins with a solvent (acetonitrile or methanol). The extract is subjected to chromatographic separation. The same extract is incubated with the enzyme for a period sufficient to observe a difference between the 2 chromatograms. The regions with the identified changes are collected and subjected to mass spectrometric analysis to determine the peptide compositions.
  - Small peptide libraries are prepared with a fluorophore at one extremity and a quencher group at the other (Meldal et al Methods in molecular biology 1998,87). The substrate can be identified using a strategy described in Apletalina et al (JBC (1998)273, 41, 26589-95). For each

hexapeptide library, the identity of one residue at one position remains constant while the rest is randomized (for a total of 6*20=120 individual libraries). Each library is made-up of 3.2 million different members and is identified both by the position of the constant residue along the hexapeptide, and its identity. The NL-enzyme is added to each library and the fluorescence is recorded. The data is organized to identify the libraries producing the most fluorescence for each position along the hexapeptide. This arrangement suggests the identity of important residues at each position along the hexapeptide. Hexapeptide representing the best suggestions are prepared and tested in a similar fashion. From this set, the hexapeptide with the best fluorescence is selected.

- set up enzymatic assays. An enzymatic assay consists in the addition of the above-identified substrate to the enzyme in constant conditions of pH, salts, temperature and time. The resulting solution is assayed for the hydrolysed peptide or for the intact peptide. This assay can be realized with specific antibodies, HPLC or, when self-quenched fluorescence tagged peptides are used (Meldal et al), by the appearance of fluorescence. The enzyme may be in solution or attached to a solid substrate;
- identify inhibitors. Inhibitors can be identified from synthetic libraries, biota extracts and from rationally designed inhibitors using X-ray crystallography and substituent activity relationships. Each molecule or extract fraction is tested for inhibitory activity using the enzymatic test described above. The molecule responsible for the largest inhibition is further tested to determine its pharmacological and toxicological properties following known procedures. The inhibitor with the best distribution, pharmacological action combined with low toxicity will be selected for drug manufacturing. Pharmaceutically acceptable formulation of the inhibitor or its acceptable salt will be prepared by mixing with known excipients to produce tablets, capsules or injectable solutions. Between 1 and 500mg of the drug is administered to the patients;
- inject the native or soluble purified NL-enzymes into subjects. In the case of disease or pathologies caused by a lack or decrease in NL activity, the purified NL could be injected intravenously or otherwise in patients. Alternatively, immobilized NL-enzymes could be introduced at the site of orthopedic surgery or implantation of devices in bones or dental tissues.

Secretion of foreign proteins and peptides

As shown in the present work, the amino-terminal domain of NL-1 (from the initiator methionine to the furin site) can be used to promote the secretion of a foreign protein (in this case the ectodomain of NL-3 and β-endorphin).

The amino-terminal domain of NL-1 but also of other naturally occurring soluble NL-enzymes could be used to:

- promote production and secretion of foreign proteins. This can be achieved by genetically fusing sequences coding for said foreign proteins downstream from and in phase with the amino-terminal of NL-1. These chimeric constructs could be introduced with the help of appropriate vectors in any of the expression systems mentioned above for protein production and secretion;
- promote production and secretion of bioactive peptides. Sequences encoding small bioactive peptides such as but not exclusively β-endorphin, the enkephalins, substance P, atrial natriuretic peptide (ANF) and osteostatine, could be fused immediately downstream and in phase the furin site of NL-1. These DNA constructs could be used as described above to produce bioactive peptides.
- serve as model to design artificial (non-naturally occurring) proteins or protein segments (protein vectors) to promote secretion of proteins or peptides. These protein vectors can be constructed to resemble a secreted protein. In this case they would be assembled of an endoplasmic reticulum signal peptide, a spacer of varying length and a furin cleavage site to which the protein or peptide destined for secretion can be fused. The total length of the spacer, furin cleavage site and protein or peptide destined for secretion must be at least 70 amino acid residues. Alternatively, such protein vectors could be assembled to resemble a type II membrane protein. In this case they would comprise from the amino to the carboxy-terminus a cytosolic domain of varying length, a transmembrane domain that also acts as a signal peptide, an extracellular segment of varying length and a furin cleavage site to which the protein or peptide destined for secretion can be fused. The total length of the extracellular segment, furin cleavage site and protein or peptide destined for secretion must be at least 70 amino acid residues.

Therapeutic applications of NL-enzymes

The inappropriate processing of endogenous peptides causes several diseases. The inappropriate processing may result from pathologic concentration of the enzyme itself, its substrate or other elements of the biochemical machinery downstream from the controlling enzyme. In this context it is possible to help the patient by managing the activity of the controlling enzyme.

- NL-enzymes have been localized to the brain and may be involved in the improper processing of β-amyloid precursor. Inhibitions of this process by drugs prepared as above, will help patients with Alzheimer disease as well as other patient suffering from diseases caused by plaque formation;
- NL-enzymes may be involved in the improper processing of other peptides involved in neurological diseases, pain or psychiatric disorders. Appropriately designed inhibitors will help in the management of such diseases;
- NL-1 is found in testis and is associated with spermatozoid maturation. Peptides improperly processed by the enzyme may lead to infertility. The addition of NL-1 ex-vivo to seminal liquid or immature spermatozoids taken directly from testis during an in-vitro fertilization procedure will increase fertility. Conversely, the use of a small-molecule inhibitor or removal of NL-1 with an antibody could increase fertility during an in-vitro fertilization procedure. The administration of a NL-1 inhibitor may increase or decrease the fertility potential. This inhibitor is formulated and administered as described above.
- NL-3 is found in ovaries and may be involved in the processing of a peptide involved in the maturation of eggs. The addition of NL-3 ex-vivo to immature eggs taken directly from ovaries during an in-vitro fertilization procedure will increase fertility. Conversely, the use of a small-molecule inhibitor or removal of NL-3 with an antibody could increase fertility during an in-vitro fertilization procedure. This inhibitor is formulated and administered as described above;
- NL-3 is found in bones. The improper processing of peptides by the enzyme may result in bone disease or abnormal phosphate metabolism. Administration of an inhibitor, as described above, will allow the disease management.

TABLE I

Extend of amino acid sequence identity between members of the NEP-like family

	hNEP	hPEX	hECE-1A	hECE-2	hKELL	sNL-1	hNL-2	hNL-3
hNEP	100*							

hPEX	35	100						
hECE-1A	39	38	100					
hECE-2	36	37	62	100				
hKELL	23	24	30	31	100			
sNL-1	55	39	39	39	26	100		
hNL-2	54	39	39	39	26	77	100	
hNL-3	35	32	37	37	28	36	34	100

*: percentage of sequence identity

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What is claimed is:

1. A neutral endopeptidase-like metallopeptidase which is found upon probing tissue nucleic acids with degenerate oligonucleotides derived from a conserved sequence located on either side of a sequence His-Glu-Xaa-Xaa-His, wherein Xaa is any amino acid, which has a sequence selected from the amino acid sequences shown in Figures 3, 4, and 5, a fragment thereof, and a variant thereof sharing at least about 80% homology with said sequence.

2. A metallopeptidase as defined in claim 1, which has the amino acid sequence shown in Figure 3.

3. A metallopeptidase as defined in claim 1, which has the amino acid sequence shown in Figure 4.

4. A metallopeptidase as defined in claim 1, which has the amino acid sequence shown in Figure 5.

5. A nucleic acid encoding the metallopeptidase of any one of claims 1 to 4.

6. A recombinant vector comprising the nucleic acid defined in claim 5.

7. A recombinant host cell expressing the nucleic acid of claim 5.

8. A method for producing a metallopeptidase as defined in any one of claims 1 to 4, which comprises the steps of culturing a recombinant host cell as defined in claim 7 in a growth supportive medium and recovering said metallopeptidase from said host cell or the culture medium.

9. A method for screening new molecules related to neutral endopeptidase (NEP), which comprises the steps of:

- aligning nucleotidic sequences of NEP and of known molecules related to NEP;
- assessing consensus sequences on either side of a sequence comprising His-Glu-Xaa-Xaa-His sequence, wherein Xaa is any amino acid;
- synthetising degenerate sequences of said consensus sequences;
- contacting said degenerate sequences with the nucleic acids of a panel of samples susceptible to express said new molecules, in conditions such that a hybridization complex can form between the nucleic acids of samples and the degenerate sequences;

- detecting the formation of said hybridization complex as an indication of a sample which comprises a molecule related to NEP; and
- sequencing the nucleic acid comprising said hybridization complex;

whereby a new sequence sharing homology with NEP is a new molecule related to NEP.

10. A method as defined in claim 9, wherein said degenerate sequences are selected from Figure 2.

11. An oligonucleotide selected from those in Figure 2.

12. A composition of matter comprising one or more of the oligonucleotides of claim 11.

13. A recombinant vector comprising a nucleic acid encoding the N-terminal part of the amino acid sequence shown in Figure 3 or 4, which N-terminal part terminates with a furin-recognition sequence.

14. A host cell transformed with the recombinant vector of claim 13.

15. A method for producing a soluble form of a membrane protein of interest having a C-terminal ectodomain, said soluble form essentially consisting of said ectodomain, which comprises:

- obtaining nucleic acids encoding essentially the ectodomain;
- fusing the nucleic acids in phase with the C-terminal end of the N-terminal part of the recombinant vector defined in claim 13;
- having the fused nucleic acids to be expressed in a host cell in the presence of a culture medium, which host cell expresses or is made to express furin; and
- recovering said soluble form in the culture medium.

16. A method as defined in claim 15, wherein said protein of interest is NL-3 or β-endorphin.

17. The soluble form of the metallopeptidase defined in any one of claims 1 to 4 which soluble form essentially consists of the ectodomain of said metallopeptidase.

18. A composition comprising the soluble metallopeptidase of claim 17.

19. An oligonucleotide derived from the nucleic acid defined in claim 4, which oligonucleotide has at least 12 nucleic acids in length.

20. An antibody directed against the metallopeptidase defined in any one of claims 1 to 4.

21. A method for detecting the presence or amount of a metallopeptidase as defined in any one of claims 1 to 4 in a sample, which comprises the steps of contacting said sample with the antibody defined in claim 20, in conditions such that an immune complex is formed between said antibody and said metallopeptidase, and detecting the presence or amount of an immune complex as an indication of the presence or amount of said metallopeptidase in said sample.

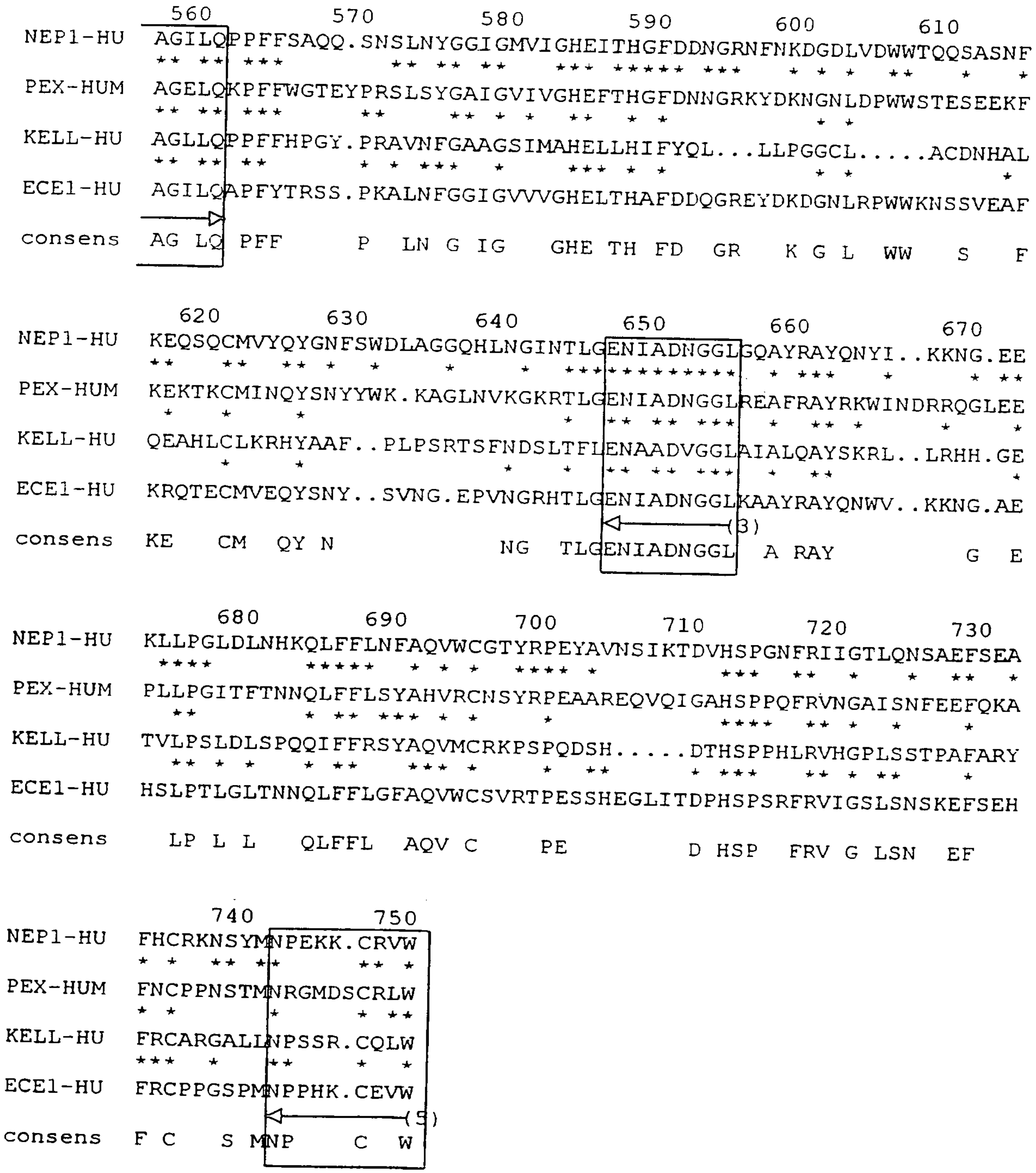
22. A method of detecting the presence or amount of a metallopeptidase as defined any one of claims 1 to 4 in a sample, which comprises the steps of: contacting said sample susceptible to comprise a target nucleic acid with the nucleic acid defined in claim 5 or with an oligonucleotide as defined in claim 19 in conditions such that a hybridization complex can form between the target nucleic acid of the sample and said nucleic acid or oligonucleotide encoding said metallopeptidase, and detecting the formation of such a hybridization complex as an indication of the presence of said metallopeptidase in said sample.

```
          1              10          20                     30
NEP1-HU   MGK.......SESQMDITDINT..PKPKKKQRWTPLEI.............SLSVLVLLL
          *                    *   *         *                      *
PEX-HUM   MEA.......ETG....SSVET..GKKANRGTRIALVV.............FVGGTLVLG
          **                   *          *                       * *
KELL-HU   MEGGDQSEEEPRERSQAGGMGTLWSQESTPEERLPVEGSRPWAV....ARRVLTAILIL.
          *                   *             *  * * **       *       *
ECE1-HU   MSTYKRATLDEEDLVDSLSEGDAYPNGLQVNFHSPRSGQRCWAARTQVEKRLVVLVVLLA

consens   M                    T            P                       L


          40        50            60        70        80        90
NEP1-HU   TIIAVTMIALYA.TYDD...GICKSSDCIKSAARLIQNMDATTEPCTDFFKYACGGWLKR
          **       *            *    **  **           **  **  ** **
PEX-HUM   TILFLVSQGLLSLQAKQ...EYCLKPECIEAAAAILSKVNLSVDPCDNFFRFACDGWISN
            * *    **           *    *       *   * ** **  ** ***
KELL-HU   .GLLLCFSVLLFYNFQNCGPRPCETSVCLDLRDHYLASGNTSVAPCTDFFSFACG...RA
           **  *   *     *   *  *    *       * *    * ** **** ***    *
ECE1-HU   AGLVACLAALGI.QYQTRSPSVCLSEACVSVTSSILSSMDPTVDPCHDFFSYACGGWIKA

consens     L      L            C    C       L      V PC DFF  ACGGW


             100       110       120         130       140       150
NEP1-HU   NVIPETSSRYGNFDILRDELEVVLKDVLQEP..KTEDIVAVQKAKALYRSCINESAIDSR
          * ***    **    **      **  *        *  * **** ** ** ** **
PEX-HUM   NPIPEDMPSYGVYPWLRHNVDLKLKELLEKSISRRRDTEAIQKAKILYSSCMNEKAIEKA
                         *       *   **             **   * *** *** *
KELL-HU   KETNNS......FQELATKNKNRLRRILEVQ.NSWHPGSGEEKAFQFYNSCMDTLAIEAA
                      *  *   *       **           * **   *  **    **
ECE1-HU   NPVPDGHSRWGTFSNLWEHNQAIIKHLLENS.TA.SVSEAERKAQVYYRACMNETRIEEL

consens   N  P      G F  L       LK  LE          A  KA   Y SCMNE AIE


               160          170       180        190       200
NEP1-HU   GGEPLLKLLPDI.YGWP..VATENWEQKYGAS.WTAEKAIAQLNSKYGKKVLINLFVGTD
             ***  *      **        *              *     *   * * * *  *
PEX-HUM   DAKPLLHILRHSPFRWPVLESNIGPEGVWSERKFSLLQTLATFRGQYSNSVFIRLYVSPD
             **                     * *    *    **     **    * * *  *
KELL-HU   GTGPLRQVIEEL........GGWRISGKWTSLNFN..RTLRLLMSQYGHFPFFRAYLGPH
             **   ** *        *** * * *   **    **      *   ***  *
ECE1-HU   RAKPLMELIERL........GGWNITGPWAKDNFQ..DTLQVVTAHYRTSPFFSVYVSAD

consens      PL                     G W    F    TL      Y    F   YV  D


                   220        230       240       250       260
NEP1-HU   DKNSVNHVIHIDQPRLGLPSR.DYYECTGIYKEACTAYVDFMISVARLIRQEERLPI.DE
          ** *  *    **  * *  * **       *    *   **   * *      *
PEX-HUM   DKASNEHILKLDQATLSLAVREDYLDNSTEAKSYRDALYKFMVDTAVL......LGA.NS
                     **         *       *   *            *      **
KELL-HU   PASPHTPVIQIDQPEFDVPLKQDQEQKI.YAQIFRE.YLTYLNQLGTL......LGG.DP
                 *** **     *       *          ** *  *** *      *** *
ECE1-HU   SKNSNSNVIQVDQSGLGLPSRDYYLNKTENEKVLTG.YLNYMVQLGKL......LGGGDE

consens    K S   VI  DQ  L LP R DY       K     Y   M     L      LG  D
```

FIG. 1

```
            270       280       290       300       310       320
NEP1-HU    NQLALEMNKVMELEKEIANATAKPEDRNDPMLLYNKMTLAQIQNNFSLEINGKPFSWLNF
                 *  *  **  **      * *       ***                 * **
PEX-HUM    SRAEHDMKSVLRLEIKIAEIMIPHENRTSEAMY.NKMNISELSAMIP......QFDWLGY
           *                     * * *           *  *  * *        ***
KELL-HU    SKVQEHSSLSISITSRLFQFLRPLEQRRAQGKLFQMVTIDQLKEMAP......AIDWLSC
                           *     * * **        **   *   **      ** **
ECE1-HU    EAIRPQMQQILDFETALANITIPQEKRRDEELIYHKVTAAELQTLAP......AINWLPF

consens          M      E   A    P E R        K T   L    P         WL


            330       340           350       360       370       380
NEP1-HU    TNEIMSTVNISITNEEDVVVYA....PEYLTKLKPILTKYSARDLQNLMSWRFIMDLVSS
                 *                   * *   *  **         *   **
PEX-HUM    IKKVIDTRLYPHLKDISPSENVVVRVPQYFKDLFRILGSERKKTIANYLVWRMVYSRIPN
                                           *                    *    *
KELL-HU    LQATFTPMSLSPSQSLVVHDVEYL...KNMSQLVEEMLLKQRDFLQSHMILGLVVTLSPA
           *   * *     *   ** * ***      * *        *  *   **  **   *
ECE1-HU    LNTIFYPVEINESEPIVVYDKEYL...EQISTLINT...TDRCLLNNYMIWNLVRKTSSF

consens                     V              L           L N M W  V


                390           400        410       420       430
NEP1-HU    LSRTYKESR....NAFRKALYGTT.SETATWRRCANYVNGNMENAVGRLYVEAAFAGESK
           ***            *     ***      *  * *         **   *   *    *
PEX-HUM    LSRRFQYRW....LEFSRVIQGTT.TLLPQWDKCVNFIESALPYVVGKMFVDVYFQEDKK
           *   **                      * * ***              **   *
KELL-HU    LDSQFQEARRKLSQKLRELTEQPPMPARPRWMKCVEETGTFFEPTLAALFVREAFGPSTR
           **  ** *      *  *          ***  **  *       *   **   *
ECE1-HU    LDQRFQDA....DEKFMEVMYGTKKTCLPRWKFCVSDTENNLGFALGPMFVKATFAEDSK

consens    L   FQ         F     GT     P W  CV           G  FV   F    K


           440       450        460       470       480       490
NEP1-HU    HVVEDLIAQIREVFIQTLD.DLTWMDAETKKRAEEKALAIKERIGYPDDIVSNDNKLNNE
              * *    *  **  *     **** **  * *** *     ***     **   *
PEX-HUM    EMMEELVEGVRWAFIDMLEKENEWMDAGTKRKAKEKARAVLAKVGYPE.FIMNDTHVNED
                *    * * *  *     **   *   *  *        *  *
KELL-HU    SAAMKLFTAIRDALITRLR.NLPWMNEETQNMAQDKVAQLQVEMGASE.WALKPELARQE
           * *      *  *    *   * ** ***   *  *        *        *
ECE1-HU    SIATEIILEIKKAFEESLS.TLKWMDEETRKSAKEKADAIYNMIGYPN.FIMDPKELDKV
                       (4)
consens         L   IR AFI  L   L WMD ET   A EKA A     GYP
                              (1A/B)


            500       510       520       530       540       550
NEP1-HU    YLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWISGAAVVNAFYSSGRNQIVFP
                  *  ** *  *  *         **  * * **      ******   *** **
PEX-HUM    LKAIKFSEADYFGNVLQTRKYLAQSDFFWLRKAVPKTEWFTNPTTVNAFYSASTNQIRFP
                         **     *            *   *   *  *** ** *     **
KELL-HU    YND.IQLGSSFLQSVLSCVRSLRARIVQSFLQPHPQHRWKVSPWDVNAYYSVSDHVVVFP
            **                                   *   *  ******      ***
ECE1-HU    FNDYTAVPDLYFENAMRFFNFSWRVTADQLRKAPNRDQWSMTPPMVNAYYSPTKNEIVFP
                                                             (2A/B)
consens              YF N               LR       W   P  VNA YS   N IVFP
```

Fig. 1 (cont'd)



```
            560       570       580       590       600       610
NEP1-HU   AGILQPPFFSAQQ.SNSLNYGGIGMVIGHEITHGFDDNGRNFNKDGDLVDWWTQQSASNF
          ** ** ***       ** ** **   *** ***** ***   * * *  **   *   *
PEX-HUM   AGELQKPFFWGTEYPRSLSYGAIGVIVGHEFTHGFDNNGRKYDKNGNLDPWWSTESEEKF
          ** ** ***     **    ** * *  **  * *          * *
KELL-HU   AGLLQPPFFHPGY.PRAVNFGAAGSIMAHELLHIFYQL...LLPGGCL.....ACDNHAL
          ** ** **      * * ***  *    *** * *          * *          *
ECE1-HU   AGILQAPFYTRSS.PKALNFGGIGVVVGHELTHAFDDQGREYDKDGNLRPWWKNSSVEAF

consens   AG LQ PFF     P  LN G IG   GHE TH FD  GR   K G L  WW   S   F

            620       630       640       650       660       670
NEP1-HU   KEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGLGQAYRAYQNYI..KKNG.EE
          **   **  ** *  *    *    *  ************  * ***   *     * **
PEX-HUM   KEKTKCMINQYSNYYWK.KAGLNVKGKRTLGENIADNGGLREAFRAYRKWINDRRQGLEE
           *   *    *                 *  ** ** ***  *  ** *     *    *
KELL-HU   QEAHLCLKRHYAAF..PLPSRTSFNDSLTFLENAADVGGLAIALQAYSKRL..LRHH.GE
               *    *             *   *  ** ** ***  *  **            *
ECE1-HU   KRQTECMVEQYSNY..SVNG.EPVNGRHTLGENIADNGGLKAAYRAYQNWV..KKNG.AE
                                                (3)
consens   KE   CM  QY N           NG  TLGENIADNGGL  A RAY         G  E

               680       690       700       710       720       730
NEP1-HU   KLLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHSPGNFRIIGTLQNSAEFSEA
           ****      *****  * * *  **** *        ***  **  *   *  **  *
PEX-HUM   PLLPGITFTNNQLFFLSYAHVRCNSYRPEAAREQVQIGAHSPPQFRVNGAISNFEEFQKA
            **       * ** *** * *    *           ****  ** *  *    *
KELL-HU   TVLPSLDLSPQQIFFRSYAQVMCRKPSPQDSH.....DTHSPPHLRVHGPLSSTPAFARY
            ** * *   * **   *** *    *  **     * ***   ** * **    *
ECE1-HU   HSLPTLGLTNNQLFFLGFAQVWCSVRTPESSHEGLITDPHSPSRFRVIGSLSNSKEFSEH

consens     LP L L   QLFFL  AQV C    PE        D HSP  FRV G LSN  EF

               740        750
NEP1-HU   FHCRKNSYMNPEKK.CRVW
          * *  ** **     ** *
PEX-HUM   FNCPPNSTMNRGMDSCRLW
          * *      *     * **
KELL-HU   FRCARGALLNPSSR.CQLW
          ***  *   **    *  *
ECE1-HU   FRCPPGSPMNPPHK.CEVW
                             (5)
consens   F C   S MNP    C  W
```

FIG. 1 (cont'd)

PRIMER	SEQUENCE
(1A)	5'-TGGATGGAT/CGA/CIGG/AIACIA/CA-3'
(1B)	5'-TGGATGGAT/CGA/CIGG/AIACIA/CG-3'
(2A)	5'-A/GTIGTITTT/CCCIGCIGGIA/GT/AIC/TTA/TCA-3'
(2B)	5'-A/GTIGTITTT/CCCIGCIGGIA/GT/AIC/TTG/CCA-3'
(3)	5'-AIICCICCIA/TC/TA/GTCIGCIG/AC/TA/GTTT/CTC-3'
(4)	5'-GAT/CAAT/CT/CTIGAT/CGAA/GT/CTIAAT/CTGGATGG-3'
(5)	5'-T/CT/CACCAIATICT/GA/GCATCG/TT/CTTCATIGGG/ATG-3'

FIG. 2

Sequence of NL-1 cDNA from mouse

```
                                                                                                       cgggagccagcaccgtgtgacctcacacccagctcag
ctgctctactccacccggagcccaccttggccagctcaccccaactctgagacatcccaacctagcctttaaggacttgcctagcagtgactgagagcaccagggtcccctgggcacttggggcacagcttacagcattgagagcag
agaccaggacagtgcaccagcttcagtgtgtcctaggcatccgatccgggctccagctgcctctctcctagccctggcctgggggcttagcggtgtgccttccacccagaaccggctgatagggaaagtctgagagcccagtgggg

                                                                                                                        30
    met val glu arg ala gly trp cys arg lys lys ser pro gly phe val glu tyr gly leu met val leu leu leu leu leu leu gly ala
  1 ATG GTG GAG AGA GCA GGC TGG TGT CGG AAG AAG TCC CCA GGC TTC GTG GAG TAT GGG CTG ATG GTG CTG CTG CTG CTG TTG CTG GGA GCC
                                                                                                                        60
    ile val thr leu gly val phe tyr ser ile gly lys gln leu pro leu leu thr ser leu leu his phe ser trp asp glu arg thr val
 91 ATA GTG ACT CTG GGT GTC TTC TAC AGC ATA GGG AAG CAG CTG CCC CTC TTA ACT AGC CTG CTA CAC TTC TCC TGG GAT GAG AGG ACG GTT
                                                                                                                        90
    val lys arg ala leu arg asp ser ser leu lys ser asp ile cys thr thr pro ser cys val ile ala ala ala arg ile leu glu asn
181 GTA AAA CGA GCC CTC AGG GAT TCA TCA CTG AAA AGT GAC ATC TGC ACC ACC CCA AGC TGT GTG ATA GCA GCT GCC AGA ATC CTC GAA AAC
                                                                                                                       120
    met asp gln ser arg asn pro cys glu asn phe tyr gln tyr ala cys gly gly trp leu arg his his val ile pro glu thr asn ser
271 ATG GAC CAA TCG AGG AAC CCC TGT GAA AAC TTC TAC CAG TAC GCC TGC GGA GGC TGG CTG AGG CAC CAC GTG ATC CCA GAG ACC AAC TCC
                                                                                                                       150
    arg tyr ser val phe asp ile leu arg asp glu leu glu val ile leu lys gly val leu glu asp ser thr ser gln his arg pro ala
361 CGA TAC AGC GTC TTT GAC ATC CTG CGG GAC GAG CTG GAG GTT ATC CTC AAA GGG GTG CTG GAG GAT TCC ACT TCC CAG CAT CGC CCG GCC
                                                                                                                       180
    val glu lys ala lys thr leu tyr arg ser cys met asn gln ser val ile glu lys arg asp ser glu pro leu leu ser val leu lys
451 GTG GAG AAG GCC AAG ACA CTA TAT CGC TCC TGC ATG AAC CAA AGT GTG ATC GAG AAG AGA GAC TCT GAG CCC CTG CTG AGC GTC TTA AAA
                                                                                                                       210
    met val gly gly trp pro val ala met asp lys trp asn glu thr met gly leu lys trp glu leu glu arg gln leu ala val leu asn
541 ATG GTA GGA GGT TGG CCT GTG GCC ATG GAT AAG TGG AAC GAG ACC ATG GGC CTC AAG TGG GAA CTG GAG CGA CAG TTG GCT GTG TTG AAC
                                                                                                                       240
    ser gln phe asn arg arg val leu ile asp leu phe ile trp asn asp asp gln asn ser ser arg his val ile tyr ile asp gln pro
631 TCG CAG TTC AAC AGG CGG GTC CTC ATC GAC CTC TTC ATC TGG AAT GAC GAC CAG AAC TCC AGC CGG CAT GTC ATC TAC ATA GAC CAG CCC
                                                                                                                       270
    thr leu gly met pro ser arg glu tyr tyr phe gln glu asp asn asn his lys val arg lys ala tyr leu glu phe met thr ser val
721 ACC TTG GGC ATG CCA TCC CGG GAG TAC TAT TTC CAG GAG GAC AAC AAC CAC AAG GTA CGG AAA GCC TAC CTG GAG TTC ATG ACG TCA GTG
```

FIG. 3

```
     ala thr met leu arg lys asp gln asn leu ser lys glu ser ala met val arg glu glu met ala glu val leu glu leu glu thr his  300
 811 GCC ACT ATG CTT AGG AAA GAC CAG AAC CTG TCC AAG GAG AGC GCC ATG GTG CGG GAG GAG ATG GCG GAG GTG CTG GAA CTG GAG ACG CAT

     leu ala asn ala thr val pro gln glu lys arg his asp val thr ala leu tyr his arg met asp leu met glu leu gln glu arg phe  330
 901 CTG GCC AAC GCC ACA GTC CCC CAG GAG AAA AGG CAT GAT GTC ACT GCC CTG TAC CAC CGA ATG GAC CTG ATG GAG CTA CAG GAA AGG TTT

     gly leu lys gly phe asn trp thr leu phe ile gln asn val leu ser ser val glu val glu leu phe pro asp glu glu val val val  360
 991 GGT CTG AAG GGG TTT AAC TGG ACT CTC TTC ATA CAA AAC GTG TTG TCT TCT GTG GAA GTC GAG CTG TTC CCA GAT GAG GAG GTG GTG GTC

     tyr gly ile pro tyr leu glu asn leu glu asp ile ile asp ser tyr ser ala arg thr met gln asn tyr leu val trp arg leu val  390
1081 TAC GGC ATC CCC TAC CTG GAG AAT CTG GAG GAT ATC ATT GAT AGC TAC TCA GCA CGG ACC ATG CAG AAC TAC CTG GTA TGG CGC CTG GTG

     leu asp arg ile gly ser leu ser gln arg phe lys glu ala arg val asp tyr arg lys ala leu tyr gly thr thr val glu glu val  420
1171 CTA GAT CGA ATT GGC AGC CTG AGC CAG AGA TTC AAA GAG GCG CGT GTG GAC TAC CGC AAG GCG CTG TAC GGC ACG ACC GTG GAG GAG GTA

     arg trp arg glu cys val ser tyr val asn ser asn met glu ser ala val gly ser leu tyr ile lys arg ala phe ser lys asp ser  450
1261 CGC TGG CGA GAG TGT GTC AGC TAT GTC AAC AGT AAC ATG GAG AGC GCC GTG GGC TCC CTC TAC ATC AAG CGG GCC TTC TCC AAG GAC AGC

     lys ser thr val arg glu leu ile glu lys ile arg ser val phe val asp asn leu asp glu leu asn trp met asp glu glu ser lys  480
1351 AAG AGC ACG GTC AGA GAG CTG ATT GAG AAG ATA AGG TCC GTG TTT GTG GAT AAC CTG GAT GAG CTG AAC TGG ATG GAC GAG GAA TCC AAG

     lys lys ala gln glu lys ala met asn ile arg glu gln ile gly tyr pro asp tyr ile leu glu asp asn asn lys his leu asp glu  510
1441 AAG AAG GCC CAG GAA AAG GCC ATG AAT ATA CGG GAA CAG ATT GGC TAC CCT GAC TAC ATT TTG GAA GAT AAC AAT AAA CAC CTG GAT GAG

     glu tyr ser ser leu thr phe tyr glu asp leu tyr phe glu asn gly leu gln asn leu lys asn asn ala gln arg ser leu lys lys  540
1531 GAA TAC TCC AGT TTG ACT TTC TAT GAG GAC CTG TAT TTT GAG AAC GGA CTT CAG AAC CTC AAG AAC AAT GCC CAG AGG AGC CTC AAG AAG

     leu arg glu lys val asp gln asn leu trp ile ile gly ala ala val val asn ala phe tyr ser pro asn arg asn gln[ile val phe  570
1621 CTT CGG GAA AAG GTG GAC CAG AAT CTC TGG ATC ATC GGG GCT GCA GTG GTC AAT GCA TTC TAC TCC CCA AAC AGA AAC CAG[ATC GTC TTT
```

FIG. 3 (cont'd)

```
                                                                                                   600
     pro ala gly ile leu gln pro pro phe phe ser lys asp gln pro gln ser leu asn phe gly gly ile gly met val ile gly his glu
1711 CCA GCA GGG ATT CTC CAG CCG CCC TTC TTC AGC AAG GAC CAA CCA CAG TCC TTG AAT TTT GGG GGC ATC GGG ATG GTG ATT GGG CAC GAG

                                                                                                   630
     ile thr his gly phe asp asp asn gly arg asn phe asp lys asn gly asn met leu asp trp trp ser asn phe ser ala arg his phe
1801 ATC ACA CAC GGC TTT GAT GAT AAT GGT CGT AAC TTT GAC AAG AAC GGC AAC ATG CTG GAC TGG TGG AGT AAC TTC TCG GCC CGG CAC TTC

                                                                                                   660
     gln gln gln ser gln cys met ile tyr gln tyr gly asn phe ser trp glu leu ala asp asn gln asn val asn gly phe ser thr leu
1891 CAA CAG CAG TCG CAA TGC ATG ATC TAT CAG TAC GGC AAC TTC TCT TGG GAA CTA GCA GAC AAC CAG AAT GTG AAC GGA TTC AGT ACC CTC

                                                                                                   690
     gly glu asn ile ala asp asn gly gly val] arg gln ala tyr lys ala tyr leu arg trp leu ala asp gly gly lys asp gln arg leu
1981 GGG GAG AAC ATT GCC GAC AAC GGA GGT GTG] CGA CAG GCA TAC AAG GCT TAC CTA CGG TGG CTG GCT GAT GGC GGC AAA GAT CAG CGA CTG

                                                                                                   720
     pro gly leu asn leu thr tyr ala gln leu phe phe ile asn tyr ala gln val trp cys gly ser tyr arg pro glu phe ala val gln
2071 CCG GGA CTG AAC CTG ACC TAT GCC CAG CTT TTC TTC ATC AAC TAT GCC CAG GTG TGG TGT GGG TCC TAT AGG CCG GAG TTC GCC GTC CAG

                                                                                                   750
     ser ile lys thr asp val his ser pro leu lys tyr arg val leu gly ser leu gln asn leu pro gly phe ser glu ala phe his cys
2161 TCC ATC AAG ACG GAC GTC CAC AGT CCT CTT AAG TAC AGG GTG CTG GGC TCA CTA CAG AAC CTG CCA GGC TTC TCT GAG GCA TTC CAC TGC
                                                             765
     pro arg gly ser pro met his pro met lys arg cys arg ile trp ***
2251 CCA CGA GGC AGC CCC ATG CAC CCC ATG AAG CGA TGT CGC ATC TGG TAG CCAAGGCTGAGCTATGCTGCGGCCCACGCCCCGCCACCCAGAGGCTTCGCGAATG

2354 GTGTAGCTGGCAGAGATGTGCAGGTCTTTGCCTGAAGGCCACCGGAGCCACCAGCCAGCCCTCCGCGCCCAGCCTAGAGTGTAGCCACCCGCCCACACCCGGGATGAGTGGTGCCGGTC

2473 CTGCGCCCCCTCAGGCCAGTGAGGGTCAGCAGCCCAGGAAGAGCAGTCAGCTGCCTTCCACCCTCTCCATAGTGTGTGGCTAAATGTTCTCGAGCTTCAGACTTGAGCTAAGTAAACGC

2925 TTC
```

FIG. 3 (cont'd)

Sequence of NL-2 cDNA from humans

```
              met val glu ser ala gly arg ala gly gln lys arg pro gly phe leu glu gly gly leu leu leu leu leu leu leu val    28
  1 GTG GGG ATG GTG GAG AGC GCC GGC CGT GCA GGG CAG AAG CGC CCG GGG TTC CTG GAG GGG GGG CTG CTG CTG CTG CTG CTG CTG GTG thr
                                                                                                                       ACC

    ala ala leu val ala leu gly val leu tyr ala asp arg arg gly lys gln leu pro arg leu ala ser arg leu cys phe leu gln    58
 91 GCT GCC CTG GTG GCC TTG GGT GTC CTC TAC GCC GAC CGC AGA GGG AAG CAG CTG CCA CGC CTT GCT AGC CGG CTG TGC TTC TTA CAG glu
                                                                                                                       GAG

    glu arg thr phe val lys arg lys pro arg gly ile pro glu ala gln glu val ser glu val cys thr thr pro gly cys val ile    88
181 GAG AGG ACC TTT GTA AAA CGA AAA CCC CGA GGG ATC CCA GAG GCC CAA GAG GTG AGC GAG GTC TGC ACC ACC CCT GGC TGC GTG ATA ala
                                                                                                                       GCA

    ala ala arg ile leu gln asn met asp pro thr thr glu pro cys asp asp phe tyr gln phe ala cys gly gly trp leu arg arg   118
271 GCC GCC AGG ATC CTC CAG AAC ATG GAC CCG ACC ACG GAA CCG TGT GAC GAC TTC TAC CAG TTT GCA TGC GGA GGC TGG CTG CGG CGC his
                                                                                                                       CAC

    val ile pro glu thr asn ser arg tyr ser ile phe asp val leu arg asp glu leu glu val ile leu lys ala val leu glu asn   148
361 GTG ATC CCT GAG ACC AAC TCA AGA TAC AGC ATC TTT GAC GTC CTC CGC GAC GAG CTG GAG GTC ATC CTC AAA GCG GTG CTG GAG AAT ser
                                                                                                                       TCG

    thr ala lys asp arg pro ala val glu lys ala arg thr leu tyr arg ser cys met asn gln ser val ile glu lys arg gly ser   178
451 ACT GCC AAG GAC CGG CCG GCT GTG GAG AAG GCC AGG ACG CTG TAC CGC TCC TGC ATG AAC CAG AGT GTG ATA GAG AAG CGA GGC TCT gln
                                                                                                                       CAG

    pro leu leu asp ile leu glu val val gly gly trp pro val ala met asp arg trp asn glu thr val gly leu glu trp glu leu   208
541 CCC CTG CTG GAC ATC TTG GAG GTG GTG GGA GGC TGG CCG GTG GCG ATG GAC AGG TGG AAC GAG ACC GTA GGA CTC GAG TGG GAG CTG glu
                                                                                                                       GAG

    arg gln leu ala leu met asn ser gln phe asn arg arg val leu ile asp leu phe ile trp asn asp asp gln asn ser ser arg   238
631 CGG CAG CTG GCG CTG ATG AAC TCA CAG TTC AAC AGG CGC GTC CTC ATC GAC CTC TTC ATC TGG AAC GAC GAC CAG AAC TCC AGC CGG his
                                                                                                                       CAC

    ile ile tyr ile asp gln pro thr leu gly met pro ser arg glu tyr tyr phe asn gly gly ser asn arg lys val arg glu ala   268
721 ATC ATC TAC ATA GAC CAG CCC ACC TTG GGC ATG CCC TCC CGA GAG TAC TAC TTC AAC GGC GGC AGC AAC CGG AAG GTG CGG GAA GCC tyr
                                                                                                                       TAC

    leu gln phe met val ser val ala thr leu leu arg glu asp ala asn leu pro arg asp ser cys leu val gln glu asp met val   298
811 CTG CAG TTC ATG GTG TCA GTG GCC ACG TTG CTG CGG GAG GAT GCA AAC CTG CCC AGG GAC AGC TGC CTG GTG CAG GAG GAC ATG GTG gln
                                                                                                                       CAG
                                                                                                                       328
```

FIG. 4

```
     val leu glu leu glu thr gln leu ala lys ala thr val pro gln glu glu arg his asp val ile ala leu tyr his arg met gly leu
 901 GTT CTG GAG CTG GAG ACA CAG CTG GCC AAG GCC ACG GTA CCC CAG GAG GAG AGA CAC GAC GTC ATC GCC TTG TAC CAC CGG ATG GGA CTG
                                                                                                                        358
     glu glu leu gln ser gln phe gly leu lys gly phe asn trp thr leu phe ile gln thr val leu ser ser val lys ile lys leu leu
 991 GAG GAG CTG CAA AGC CAG TTT GGC CTG AAG GGA TTT AAC TGG ACT CTG TTC ATA CAA ACT GTG CTA TCC TCT GTC AAA ATC AAG CTG CTG
                                                                                                                        388
     pro asp glu glu val val val tyr gly ile pro tyr leu gln asn leu glu asn ile ile asp thr tyr ser ala arg thr ile gln asn
1081 CCA GAT GAG GAA GTG GTG GTC TAT GGC ATC CCC TAC CTG CAG AAC CTT GAA AAC ATC ATC GAC ACC TAC TCA GCC AGG ACC ATA CAG AAC
                                                                                                                        418
     tyr leu val trp arg leu val leu asp arg ile gly ser leu ser gln arg phe lys asp thr arg val asn tyr arg lys ala leu phe
1171 TAC CTG GTC TGG CGC CTG GTG CTG GAC CGC ATT GGT AGC CTA AGC CAG AGA TTC AAG GAC ACA CGA GTG AAC TAC CGC AAG GCG CTG TTT
                                                                                                                        448
     gly thr met val glu glu val arg trp arg glu cys val gly tyr val asn ser asn met glu asn ala val gly ser leu tyr val arg
1261 GGC ACA ATG GTG GAG GAG GTG CGC TGG CGT GAA TGT GTG GGC TAC GTC AAC AGC AAC ATG GAG AAC GCC GTG GGC TCC CTC TAC GTC AGG
                                                                                                                        478
     glu ala phe pro gly asp ser lys ser met val arg glu leu ile asp lys val arg thr val phe val glu thr leu asp glu leu gly
1351 GAG GCG TTC CCT GGA GAC AGC AAG AGC ATG GTC AGA GAA CTC ATT GAC AAG GTG CGG ACA GTG TTT GTG GAG ACG CTG GAC GAG CTG GGC
                                                                                                                        508
     trp met asp glu glu ser lys lys lys ala gln glu lys ala met ser ile arg glu gln ile gly his pro asp tyr ile leu glu glu
1441 TGG ATG GAC GAG GAG TCC AAG AAG AAG GCG CAG GAG AAG GCC ATG AGC ATC CGG GAG CAG ATC GGG CAC CCT GAC TAC ATC CTG GAG GAG
                                                                                                                        538
     met asn arg arg leu asp glu glu tyr ser asn leu asn phe ser glu asp leu tyr phe glu asn ser leu gln asn leu lys val gly
1531 ATG AAC AGG CGC CTG GAC GAG GAG TAC TCC AAT CTG AAC TTC TCA GAG GAC CTG TAC TTT GAG AAC AGT CTG CAG AAC CTC AAG GTG GGC
                                                                                                                        568
     ala gln arg ser leu arg lys leu arg glu lys val asp pro asn leu trp ile ile gly ala ala val val asn ala phe tyr ser pro
1621 GCC CAG CGG AGC CTC AGG AAG CTT CGG GAA AAG GTG GAC CCA AAT CTC TGG ATC ATC GGG GCG GCG GTG GTC AAT GCG TTC TAC TCC CCA
                                                                                                                        598
     asn arg asn gln ile val phe pro ala gly ile leu gln pro pro phe phe ser lys glu gln pro gln ala leu asn phe gly gly ile
1711 AAC CGA AAC CAG ATT GTA TTC CCT GCC GGG ATC CTC CAG CCC CCC TTC TTC AGC AAG GAG CAG CCA CAG GCC TTG AAC TTT GGA GGC ATT
                                                                                                                        628
```

FIG - 4 (cont'd)

```
     gly met val ile gly his glu ile thr his gly phe asp asp asn gly arg asn phe asp lys asn gly asn met met asp trp trp ser
1801 GGG ATG GTG ATC GGG CAC GAG ATC ACG CAC GGC TTT GAC GAC AAT GGC CGG AAC TTC GAC AAG AAT GGC AAC ATG ATG GAT TGG TGG AGT
                                                                                                                         658
     asn phe ser thr gln his phe arg glu gln ser glu cys met ile tyr gln tyr gly asn tyr ser trp asp leu ala asp glu gln asn
1891 AAC TTC TCC ACC CAG CAC TTC CGG GAG CAG TCA GAG TGC ATG ATC TAC CAG TAC GGC AAC TAC TCC TGG GAC CTG GCA GAC GAA CAG AAC
                                                                                                                         688
     val asn gly phe asn thr leu gly glu asn ile ala asp asn gly gly val arg gln ala tyr lys ala tyr leu lys trp met ala glu
1981 GTG AAC GGA TTC AAC ACC CTT GGG GAA AAC ATT GCT GAC AAC GGA GGG GTG CGG CAA GCC TAT AAG GCC TAC CTC AAG TGG ATG GCA GAG
                                                                                                                         718
     gly gly lys asp gln gln leu pro gly leu asp leu thr his glu gln leu phe phe ile asn tyr ala gln val trp cys gly ser tyr
2071 GGT GGC AAG GAC CAG CAG CTG CCC GGC CTG GAT CTC ACC CAT GAG CAG CTC TTC TTC ATC AAC TAT GCC CAG GTG TGG TGC GGG TCC TAC
                                                                                                                         748
     arg pro glu phe ala ile gln ser ile lys thr asp val his ser pro leu lys tyr arg val leu gly ser leu gln asn leu ala ala
2161 CGG CCC GAG TTC GCC ATC CAA TCC ATC AAG ACA GAC GTC CAC AGT CCC CTG AAG TAC AGG GTA CTG GGG TCG CTG CAG AAC CTG GCC GCC
                                                                                 770
     phe ala asp thr phe his cys ala arg gly thr pro met his pro lys glu arg cys arg val trp ter
2251 TTC GCA GAC ACG TTC CAC TGT GCC CGG GGC ACC CCC ATG CAC CCC AAG GAG CGA TGC CGC GTG TGG TAG CCA AGG CCC TGC CGC GCT GTG

2341 CGG CCC ACG CCC ACC CGC TGC TCG GAG GCA TCT GTG CGA AGG TGC AGC TAG CGG CGA CCC AGT GTA CGT CCC GCC CCG GCC AAC CAT GCC

2431 AAG CCT GCC TGC CAG GCC TCT GCG CCT GGC CTA GGG TGC AGC CAC CTG CCT GAC ACC CAG GGA TGA GCA GTG TCC AGT GCA GTA CCT GGA

2521 CCG GAG CCC CCT TCA CAG ACA CCC GCG GGG CTC AGT GCC CCC GTC ACA ACT CTG TAG AGA CAA TCA ACT GTG TCC TGC CCA CCC TTC AAG

2611 GTG CAT TGT CTT CCA GTA TCT ACA GCT TCA GAA CTT GAG CTA AGT AAA TGC TTT CAA AGA AAA AAA
```

FIG. 4 (cont'd)

Sequence of NL-3 cDNA from human

```
☐    1 GGC GCT GGG AGA CAC CGG ACG CCC GCT CGG CTG CGC TGC GGC TCA GGC CCC CGC TCG GCC CGA CCC GCT CGG TCA CCG CCG GCT CGG GCG
☐
☐   91 CGC ACC TGC CGG CTG CGG CCC CAG GGC CAT GCG GAG GCC CAC GAG GAG GCC GGC GGC CAC GCG CAT CCC GTA GCC CAG GTG GCC CAG GTC
                                                                                                                            22
                                       met glu pro pro tyr ser leu thr ala his tyr asp glu phe gln glu val lys tyr val ser arg
    181 TGC ACC GCG GCG GCC TCG GCG CCG ATG GAG CCC CCG TAT TCG CTG ACG GCG CAC TAC GAT GAG TTC CAA GAG GTC AAG TAC GTG AGC CGC
                                                                                                                            52
        cys gly ala gly gly ala arg gly ala ser leu pro pro gly phe pro leu gly ala ala arg ser ala thr gly ala arg ser gly leu
    271 TGC GGC GCG GGG GGC GCG CGC GGG GCC TCC CTG CCC CCG GGC TTC CCG TTG GGC GCT GCG CGC AGC GCC ACC GGG GCC CGG TCC GGG CTG
                                                                                                                            82
        pro arg trp asn arg arg glu val cys leu leu ser gly leu val phe ala ala gly leu cys ala ile leu ala ala met leu ala leu
    361 CCG CGC TGG AAC CGG CGC GAG GTG TGC CTG CTG TCG GGG CTG GTG TTC GCC GCC GGC CTC TGC GCC ATT CTG GCG GCT ATG CTG GCC CTC
                                                                                                                           112
        lys tyr leu gly pro val ala ala gly gly gly ala cys pro glu gly cys pro glu arg lys ala phe ala arg ala ala arg phe leu
    451 AAG TAC CTG GGC CCG GTC GCG GCC GGC GGC GGC GCC TGT CCC GAG GGC TGC CCT GAG CGC AAG GCC TTC GCG CGC GCC GCT CGC TTC CTG
                                                                                                                           142
        ala ala asn leu asp ala ser ile asp pro cys gln asp phe tyr ser phe ala cys gly gly trp leu arg arg his ala ile pro asp
    541 GCC GCC AAC CTG GAC GCC AGC ATC GAC CCA TGC CAG GAC TTC TAC TCG TTC GCC TGC GGC GGT TGG CTG CGG CGC CAC GCC ATC CCC GAC
                                                                                                                           172
        asp lys leu thr tyr gly thr ile ala ala ile gly glu gln asn glu glu arg leu arg arg leu leu ala arg pro gly gly gly pro
    631 GAC AAG CTC ACC TAT GGC ACC ATC GCG GCA ATC GGC GAG CAA AAC GAG GAG CGC CTA CGG CGC CTG CTG GCG CGG CCC GGG GGT GGG CCT
                                                                                                                           202
        gly gly ala ala gln arg lys val arg ala phe phe arg ser cys leu asp met arg glu ile glu arg leu gly pro arg pro met leu
    721 GGC GGC GCG GCC CAG CGC AAG GTG CGC GCC TTC TTC CGC TCG TGC CTC GAC ATG CGC GAG ATC GAG CGA CTG GGC CCG CGA CCC ATG CTA
                                                                                                                           232
        glu val ile glu asp cys gly gly trp asp leu gly gly ala glu glu arg pro gly val ala ala arg trp asp leu asn arg leu leu
    811 GAG GTC ATC GAG GAC TGC GGG GGC TGG GAC CTG GGC GGC GCG GAG GAG CGT CCG GGG GTC GCG GCG CGA TGG GAC CTC AAC CGG CTG CTG
                                                                                                                           262
        tyr lys ala gln gly val tyr ser ala ala ala leu phe ser leu thr val ser leu asp asp arg asn ser ser arg tyr val ile arg
    901 TAC AAG GCG CAG GGC GTG TAC AGC GCC GCC GCG CTC TTC TCG CTC ACG GTC AGC CTG GAC GAC AGG AAC TCC TCG CGC TAC GTC ATC CGC
                                                                                                                           292
        ile asp gln asp gly leu thr leu pro glu arg thr leu tyr leu ala gln asp glu asp ser glu lys val leu ala ala tyr arg val
    991 ATT GAC CAG GAT GGG CTC ACC CTG CCA GAG AGG ACC CTG TAC CTC GCT CAG GAT GAG GAC AGT GAG AAG GTC CTG GCA GCA TAC AGG GTG
                                                                                                                           322
        phe met glu arg val leu ser leu leu gly ala asp ala val glu gln lys ala gln glu ile leu gln val glu gln gln leu ala asn
   1081 TTC ATG GAG CGA GTG CTC AGC CTC CTG GGT GCA GAC GCT GTG GAA CAG AAG GCC CAA GAG ATC CTG CAA GTG GAG CAG CAG CTG GCC AAC
```

FIG. 5

```
                                                                                                                                        352
     ile thr val ser glu tyr asp asp leu arg arg asp val ser ser met tyr asn lys val thr leu gly gln leu gln lys ile thr pro
1171 ATC ACT GTG TCA GAG TAT GAC GAC CTA CGG CGA GAT GTC AGC TCC ATG TAC AAC AAG GTG ACG CTG GGG CAG CTG CAG AAG ATC ACC CCC
                                                                                                                        382
     his leu arg trp lys trp leu leu asp gln ile phe gln glu asp phe ser glu glu glu glu val val leu leu ala thr asp tyr met
1261 CAC TTG CGG TGG AAG TGG CTG CTA GAC CAG ATC TTC CAG GAG GAC TTC TCA GAG GAA GAG GAG GTG GTG CTG CTG GCG ACA GAC TAC ATG
                                                                                                                        412
     gln gln val ser gln leu ile arg ser thr pro his arg val leu his asn tyr leu val trp arg val val val val leu ser glu his
1351 CAG CAG GTG TCG CAG CTC ATC CGC TCC ACA CCC CAC CGG GTC CTG CAC AAC TAC CTG GTG TGG CGC GTG GTG GTG GTC CTG AGT GAA CAC
                                                                                                                        442
     leu ser pro pro phe arg glu ala leu his glu leu ala gln glu met glu gly ser asp lys pro gln glu leu ala arg val cys leu
1441 CTG TCC CCG CCA TTC CGT GAG GCA CTG CAC GAG CTG GCA CAG GAG ATG GAG GGC AGC GAC AAG CCA CAG GAG CTG GCC CGG GTC TGC TTG
                                                                                                                        472
     gly gln ala asn arg his phe gly met ala leu gly ala leu phe val his glu his phe ser ala ala ser lys ala lys val gln gln
1531 GGC CAG GCC AAT CGC CAC TTT GGC ATG GCG CTT GGC GCC CTC TTT GTA CAT GAG CAC TTC TCA GCT GCC AGC AAA GCC AAG GTG CAG CAG
                                                                                                                        502
     leu val glu asp ile lys tyr ile leu gly gln arg leu glu glu leu asp trp met asp ala glu thr arg ala ala ala arg ala lys
1621 CTA GTG GAA GAC ATC AAG TAC ATC CTG GGC CAG CGC CTG GAG GAG CTG GAC TGG ATG GAC GCC GAG ACC AGG GCT GCT GCT CGG GCC AAG
                                                                                                                        532
     leu gln tyr met met val met val gly tyr pro asp phe leu leu lys pro asp ala val asp lys glu tyr glu phe glu val his glu
1711 CTC CAG TAC ATG ATG GTG ATG GTC GGC TAC CCG GAC TTC CTG CTG AAA CCC GAT GCT GTG GAC AAG GAG TAT GAG TTT GAG GTC CAT GAG
                                                                                                                        562
     lys thr tyr phe lys asn ile leu asn ser ile arg phe ser ile gln leu ser val lys lys ile arg gln glu val asp lys ser thr
1801 AAG ACC TAC TTC AAG AAC ATC TTG AAC AGC ATC CGC TTC AGC ATC CAG CTC TCA GTT AAG AAG ATT CGG CAG GAG GTG GAC AAG TCC ACG
                                                                                                                        592
     trp leu leu pro pro gln ala leu asn ala tyr tyr leu pro asn lys asn gln met val phe pro ala gly ile leu gln pro thr leu
1891 TGG CTG CTC CCC CCA CAG GCG CTC AAT GCC TAC TAT CTA CCC AAC AAG AAC CAG ATG GTG TTC CCC GCG GGC ATC CTG CAG CCC ACC CTG
                                                                                                                        622
     tyr asp pro asp phe pro gln ser leu asn tyr gly gly ile gly thr ile ile gly his glu leu thr his gly tyr asp asp trp gly
1981 TAC GAC CCT GAC TTC CCA CAG TCT CTC AAC TAC GGG GGC ATC GGC ACC ATC ATT GGA CAT GAG CTG ACC CAC GGC TAC GAC GAC TGG GGG
                                                                                                                        652
```

FIG. 5 (cont'd)

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```
     gly gln tyr asp arg ser gly asn leu leu his trp trp thr glu ala ser tyr ser arg phe leu arg lys ala glu cys ile val arg
2071 GGC CAG TAT GAC CGC TCA GGG AAC CTG CTG CAC TGG TGG ACG GAG GCC TCC TAC AGC CGC TTC CTG CGA AAG GCT GAG TGC ATC GTC CGT
                                                                                                                         682
     leu tyr asp asn phe thr val tyr asn gln arg val asn gly lys his thr leu gly glu asn ile ala asp met gly gly leu lys leu
2161 CTC TAT GAC AAC TTC ACT GTC TAC AAC CAG CGG GTG AAC GGG AAA CAC ACG CTT GGG GAG AAC ATC GCA GAT ATG GGC GGC CTC AAG CTG
                                                                                                                         712
     ala tyr his ala tyr gln lys trp val arg glu his gly pro glu his pro leu pro arg leu lys tyr thr his asp gln leu phe phe
2251 GCC TAC CAC GCC TAT CAG AAG TGG GTG CGG GAG CAC GGC CCA GAG CAC CCA CTT CCC CGG CTC AAG TAC ACA CAT GAC CAG CTC TTC TTC
                                                                                                                         742
     ile ala phe ala gln asn trp cys ile lys arg arg ser gln ser ile tyr leu gln val leu thr asp lys his ala pro glu his tyr
2341 ATT GCC TTT GCC CAG AAC TGG TGC ATC AAG CGG CGG TCG CAG TCC ATC TAC CTG CAG GTG CTG ACT GAC AAG CAT GCC CCT GAG CAC TAC
                                                                                                                         772
     arg val leu gly ser val ser gln phe glu glu phe gly arg val leu his cys pro lys val ser pro met asn pro ala his lys cys
2431 AGG GTG CTG GGC AGT GTG TCC CAG TTT GAG GAG TTT GGC CGG GTT TTA CAC TGT CCA AAG GTC TCA CCC ATG AAC CCT GCC CAC AAG TGT
             775
     ser val trp ter
2521 TCC GTG TGG TGA CCC TGG CTG CCC GCC TGC ACG CCC CCA CTG CCC CCG CAC GAA TCA CCT CCT GCT GGC TAC CGG GGC AGG CAT GCA CCC

2611 GGT GCC AGC CCC GCT CTG GGC ACC ACC TGC CTT CCA GCC CCT CCA GGA CCC GGT CCC CCT GCT GCC CCT CAC TTC AGG AGG GGC CTG GAG

2701 CAG GGT GAG GCT GGA CTT TGG GGG GCT GTG AGG GAA ATA TAC TGG GGT CCC CAG ATT CTG CTC TAA GGG GGC CAG ACC CTC TGC CAG GCT

2791 GGA TTG TAC GGG CCC CAC CTT CGC TGT GTT CTT GCT GCA AGT CTG GTC AAA TAA ATC ACT GCA CTG TTA AAA AAA AAA AAA
```

FIG. 5 (cont'd)

Sequence comparison between NEP, NL1, NL2 and NL3

```
         1                   10        20        30          40
NEP-HUM  MG............KSESQMDITDINTPKPKKKQRWTPLEISLSVLVLL..LTII...AV
         *                                     *      *        *
NL1-MOU  MV............ERAGWCRKKSPGFVEYGLMVLLLLLLGAIVTLG.V..FYSI.GKQL
         **            * **    * *** * **  ****   * * **         ****
NL2-HUM  MV............ESAGRAGQKRPGFLEGGLLLLLLLVTAALVALGVL..YADRRGKQL
         *                        *   *  *       **  * *
NL3-HUM  MEPPYSLTAHYDEFQEVKYVSRCGAGGARGASLPPGFPLGAARSATGARSGLPRWNRREV


                    50                           60        70
NEP-HUM  TMIA.......LYATYDD..................GICKSSDCIKSAARLIQ.NMDATT
                    *    *                    **    *   ***    ***
NL1-MOU  PLLTSL.....LHFSWDERTVVKR...ALRDSSLKSDICTTPSCVIAAARILE.NMDQSR
         * * *      * *   *** ***           *  **** *********  ***
NL2-HUM  PRLASR.....LCFLQEERTFVKRKPRGIPEAQEVSEVCTTPGCVIAAARILQ.NMDPTT
           *        **         *             * *        *** *  * *
NL3-HUM  CLLSGLVFAAGLCAILAAMLALKYLGPVAAGGGACPEGCPERKAFARAARFLAANLDASI


          80        90       100       110       120       130
NEP-HUM  EPCTDFFKYACGGWLKRNVIPETSSRYGNFDILRDELEVVLKDVLQEPKTEDIVAVQ.KA
          **  *  *******   ***** ***  ********** ** **         **  **
NL1-MOU  NPCENFYQYACGGWLRHHVIPETNSRYSVFDILRDELEVILKGVLEDSTSQHRPAVE.KA
          **  *** ******* *********** ** ********** *** **   ***** **
NL2-HUM  EPCDDFYQFACGGWLRRHVIPETNSRYSIFDVLRDELEVILKAVLENSTAKDRPAVE.KA
          ** *** ********** **     *          *  *   *         *   *
NL3-HUM  DPCQDFYSFACGGWLRRHAIPDDKLTYGTIAAIGEQNEERLRRLLARPGGGPGGAAQRKV


          140       150       160       170       180       190
NEP-HUM  KALYRSCINESAIDSRGGEPLLKLLPDIYGWPVATENWEQKYGASWTAEKAIAQLNSKYG
         * ***** * * *  *  ****  *    *****   *    *  *  *   * ***
NL1-MOU  KTLYRSCMNQSVIEKRDSEPLLSVLKMVGGWPVAMDKWNETMGLKWELERQLAVLNSQFN
          *************** * ***  *  ********* **** ** ********  *****
NL2-HUM  RTLYRSCMNQSVIEKRGSQPLLDILEVVGGWPVAMDRWNETVGLEWELERQLALMNSQFN
         *   ***     **  *  * *   *  ***          *   * * * *
NL3-HUM  RAFFRSCLDMREIERLGPRPMLEVIEDCGGWDLGGAEERPGVAARWDLNRLLYKAQGVYS


          200       210       220       230       240       250
NEP-HUM  KKVLINLFVGTDDKNSVNHVIHIDQPRLGLPSRDYYECTGIYKEACTAYVDFMISVARLI
           *** **   ** **  *** **** ** *** **           **  ** ***
NL1-MOU  RRVLIDLFIWNDDQNSSRHVIYIDQPTLGMPSREYYFQEDNNHKVRKAYLEFMTSVATML
         ******************* *****************    * *** *** ** **** *
NL2-HUM  RRVLIDLFIWNDDQNSSRHIIYIDQPTLGMPSREYYFNGGSNRKVREAYLQFMVSVATLL
            *  *    ** ****  * ***  *  * *  *                 *     *
NL3-HUM  AAALFSLTVSLDDRNSSRYVIRIDQDGLTLPERTLYLAQDEDSE..KVLAAYRVFMERVL
```

FIG. 6

```
             260       270       280       290       300       310
NEP-HUM    RQEERLPIDENQLALEMNKVMELEKEIANATAKP..EDRNDPMLLYNKMTLAQIQNNFSL
           *    *         **  * ***   ****     * * *   **  * *   *  * *
NL1-MOU    RKDQNLSKESAMVREEMAEVLELETHLANATVPQ..EKRHDVTALYHRMDLMELQERFGL
           * * **   *  * * *  ****** ** *****  * **** ****** * ***  ***
NL2-HUM    REDANLPRDSCLVQEDMVQVLELETQLAKATVPQ..EERHDVIALYHRMGLEELQSQFGL
                *  *           *  * ***  **      * **   *    *  **
NL3-HUM    SL...LGADAV..EQKAQEILQVEQQLANITVSEYDDLRRDVSSMYNKVTLGQLQKITP.


             320       330       340       350       360       370
NEP-HUM    EINGKPFSWLNFTNEIMSTVNISITNEEDVVVYAPEYLTKLKPILTKYSARDLQNLMSWR
               * * *  *     * *       * ****   **  *  *   ****  **   **
NL1-MOU    ....KGFNWTLFIQNVLSSVEVELFPDEEVVVYGIPYLENLEDIIDSYSARTMQNYLVWR
               ********** *****   * ************* *** *** ***** *******
NL2-HUM    ....KGFNWTLFIQTVLSSVKIKLLPDEEVVVYGIPYLQNLENIIDTYSARTIQNYLVWR
                   *                  ****     * *     *     *   ******
NL3-HUM    .....HLRWKWLLDQIF...QEDFSEEEEVVLLATDYMQQVSQLIRSTPHRVLHNYLVWR


             380       390       400       410       420       430
NEP-HUM    FIMDLVSSLSRTYKESRNAFRKALYGTTSETATWRRCANYVNGNMENAVGRLYVEAAFAG
              *   ***   ** *   ******** *   ** *  *** *** *** **   **
NL1-MOU    LVLDRIGSLSQRFKEARVDYRKALYGTTVEEVRWRECVSYVNSNMESAVGSLYIKRAFSK
           **************  ** ***** ** ********** ******* ******   **
NL2-HUM    LVLDRIGSLSQRFKDTRVNYRKALFGTMVEEVRWRECVGYVNSNMENAVGSLYVREAFPG
            *      **  *            *        * * *  *     * * * * * *
NL3-HUM    VVVVLSEHLSPPFREALHELAQEMEGSDKPQELARVCLGQANRHFGMALGALFVHEHFSA


             440       450       460       470       480       490
NEP-HUM    ESKHVVEDLIAQIREVFIQTLDDLTWMDAETKKRAEEKALAIKERIGYPDDIVSNDNK.L
            **  *  **  ** **   ** * *** * ** * ***  * * ***** *    ** *
NL1-MOU    DSKSTVRELIEKIRSVFVDNLDELNWMDEESKKKAQEKAMNIREQIGYPDYILEDNNKHL
           **** ***** * * ***  **** *************** ****** ******  *  *
NL2-HUM    DSKSMVRELIDKVRTVFVETLDELGWMDEESKKKAQEKAMSIREQIGHPDYILEEMNRRL
            **  *  *           * ** *** *    *  *        * **  *
NL3-HUM    ASKAKVQQLVEDIKYILGQRLEELDWMDAETRAAARAKLQYMMVMVGYPDFLLKPDA..V


             500       510       520       530       540       550
NEP-HUM    NNEYLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWISGAAVVNAFYSSGRNQI
             **  *   ** ****  ****      *********   ** **********  ****
NL1-MOU    DEEYSSLTFYEDLYFENGLQNLKNNAQRSLKKLREKVDQNLWIIGAAVVNAFYSPNRNQI
           ***** * * ******* *****  ***** ******* *********************
NL2-HUM    DEEYSNLNFSEDLYFENSLQNLKVGAQRSLRKLREKVDPNLWIIGAAVVNAFYSPNRNQI
           * *       *  ** * *       * *  * *  **   *       ** * ** **
NL3-HUM    DKE.YEFEVHEKTYFKNILNSIRFSIQLSVKKIRQEVDKSTWLLPPQALNAYYLPNKNQM
```

FIG. 6 (cont'd)

```
              560       570       580       590       600       610
NEP-HUM   VFPAGILQPPFFSAQQSNSLNYGGIGMVIGHEITHGFDDNGRNFNKDGDLVDWWTQQSAS
          *************  *  *** ********************** * *   ***   **
NL1-MOU   VFPAGILQPPFFSKDQPQSLNFGGIGMVIGHEITHGFDDNGRNFDKNGNMLDWWSNFSAR
          ************** *** ******************************* *******
NL2-HUM   VFPAGILQPPFFSKEQPQALNFGGIGMVIGHEITHGFDDNGRNFDKNGNMMDWWSNFSTQ
          *********       ** ** ****  **** *** ** *   *  **   **   *
NL3-HUM   VFPAGILQPTLYDPDFPQSLNYGGIGTIIGHELTHGYDDWGGQYDRSGNLLHWWTEASYS

              620       630       640       650       660       670
NEP-HUM   NFKEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGLGQAYRAYQNYIKKNGEEK
           *  ***** ******** **  *  **  ***********  *** **       *
NL1-MOU   HFQQQSQCMIYQYGNFSWELADNQNVNGFSTLGENIADNGGVRQAYKAYLRWLADGGKDQ
          **  ** ******** ** *** ****** ******************** * * *****
NL2-HUM   HFREQSECMIYQYGNYSWDLADEQNVNGFNTLGENIADNGGVRQAYKAYLKWMAEGGKDQ
           *    **    * *        * ***  ******** **   ** ** **  * *
NL3-HUM   RFLRKAECIVRLYDNFT...VYNQRVNGKHTLGENIADMGGLKLAYHAYQKWVREHGPEH

              680       690       700       710       720       730
NEP-HUM   LLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVHSPGNFRIIGTLQNSAEFSEAF
           **** *   **** * ****** **** ** *********   *  * ***   *****
NL1-MOU   RLPGLNLTYAQLFFINYAQVWCGSYRPEFAVQSIKTDVHSPLKYRVLGSLQNLPGFSEAF
           **** **  ******************** **********************  *   *
NL2-HUM   QLPGLDLTHEQLFFINYAQVWCGSYRPEFAIQSIKTDVHSPLKYRVLGSLQNLAAFADTF
           ** *  ** *****  ** **   *         ** * *  ******      *
NL3-HUM   PLPRLKYTHDQLFFIAFAQNWCIKRRSQSIYLQVLTDKHAPEHYRVLGSVSQFEEFGRVL

              740       750
NEP-HUM   HCRKNSYMNPEKKCRVW
          **   * * * * ** *
NL1-MOU   HCPRGSPMHPMKRCRIW
          ** ** ****  *** *
NL2-HUM   HCARGTPMHPKERCRVW
          **    ** *   * **
NL3-HUM   HCPKVSPMNPAHKCSVW
```

FIG. 6 (cont'd)

NL1 in the TESTIS

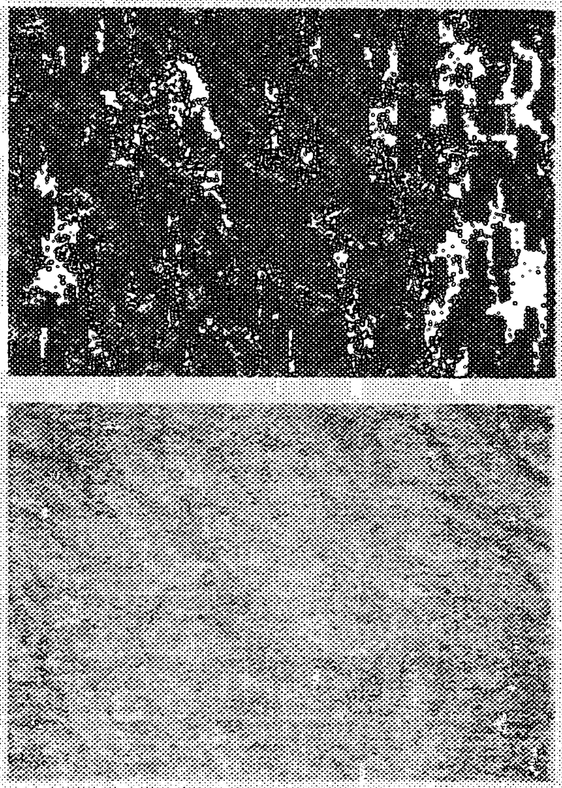


FIG. 7

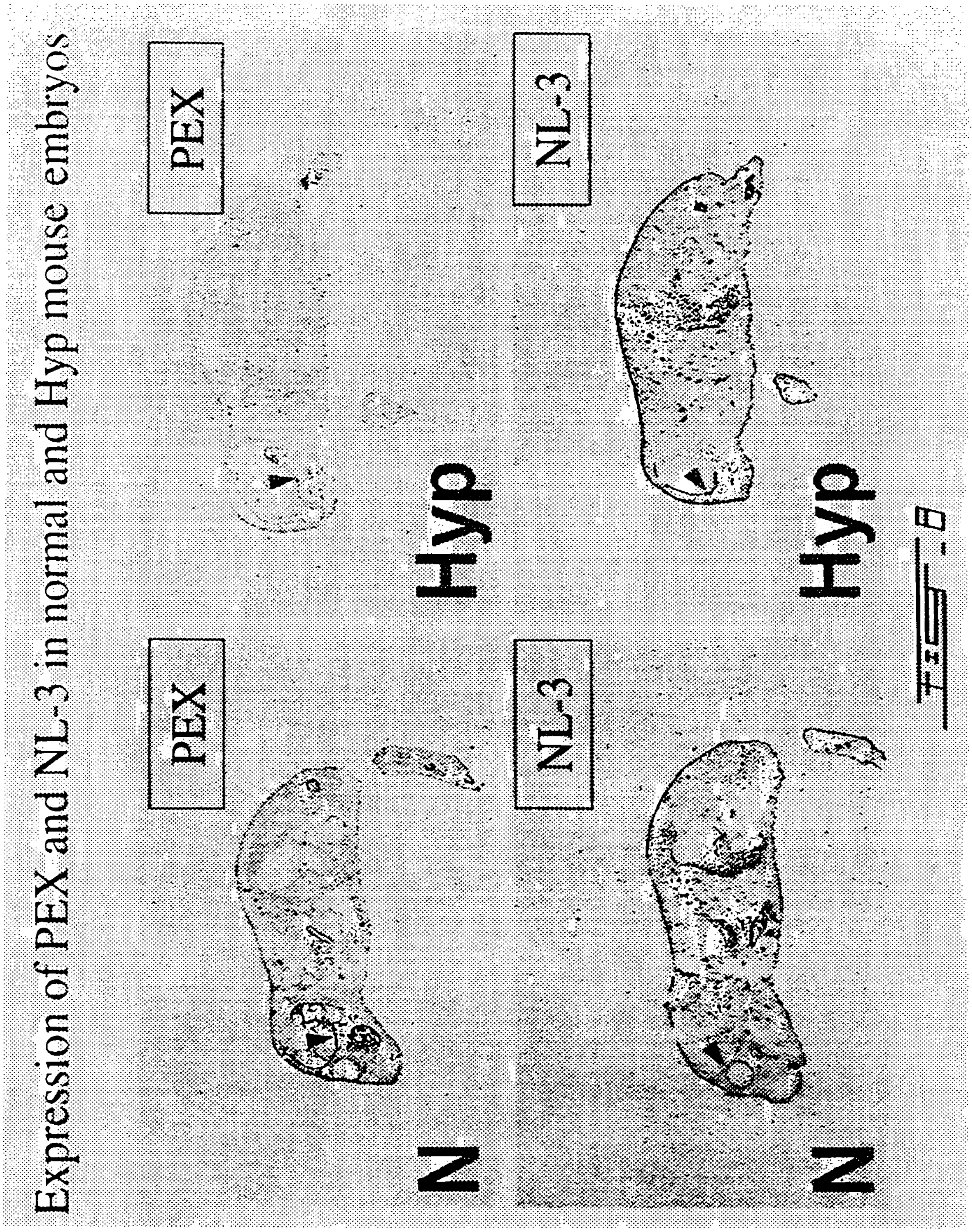
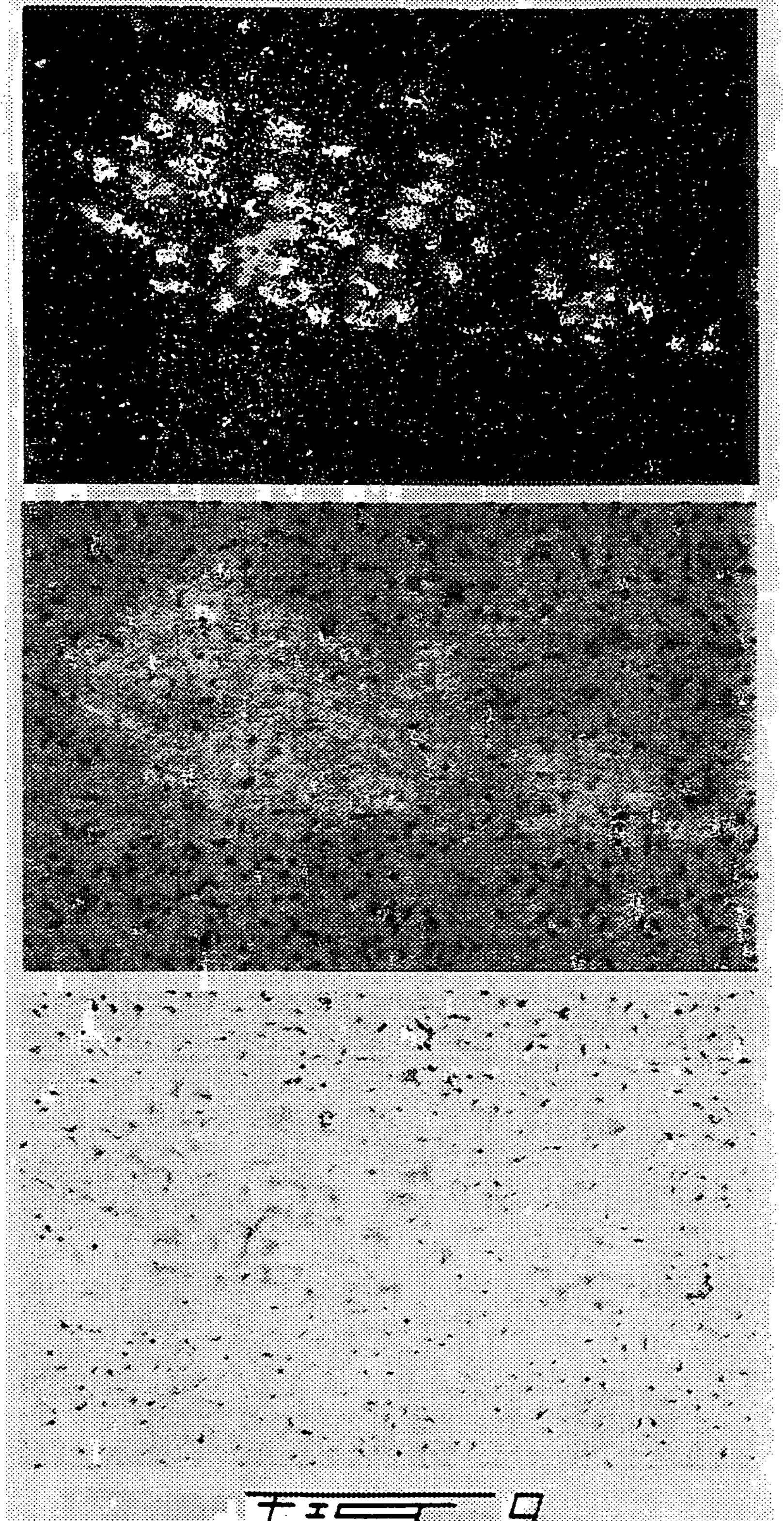

Expression of PEX and NL-3 in normal and Hyp mouse embryos
N
PEX
Hyp
PEX
N
NL-3
Hyp
NL-3

Fig-8



NL3 in the BRAIN

FIG. 9

Structure and expression of NL-1

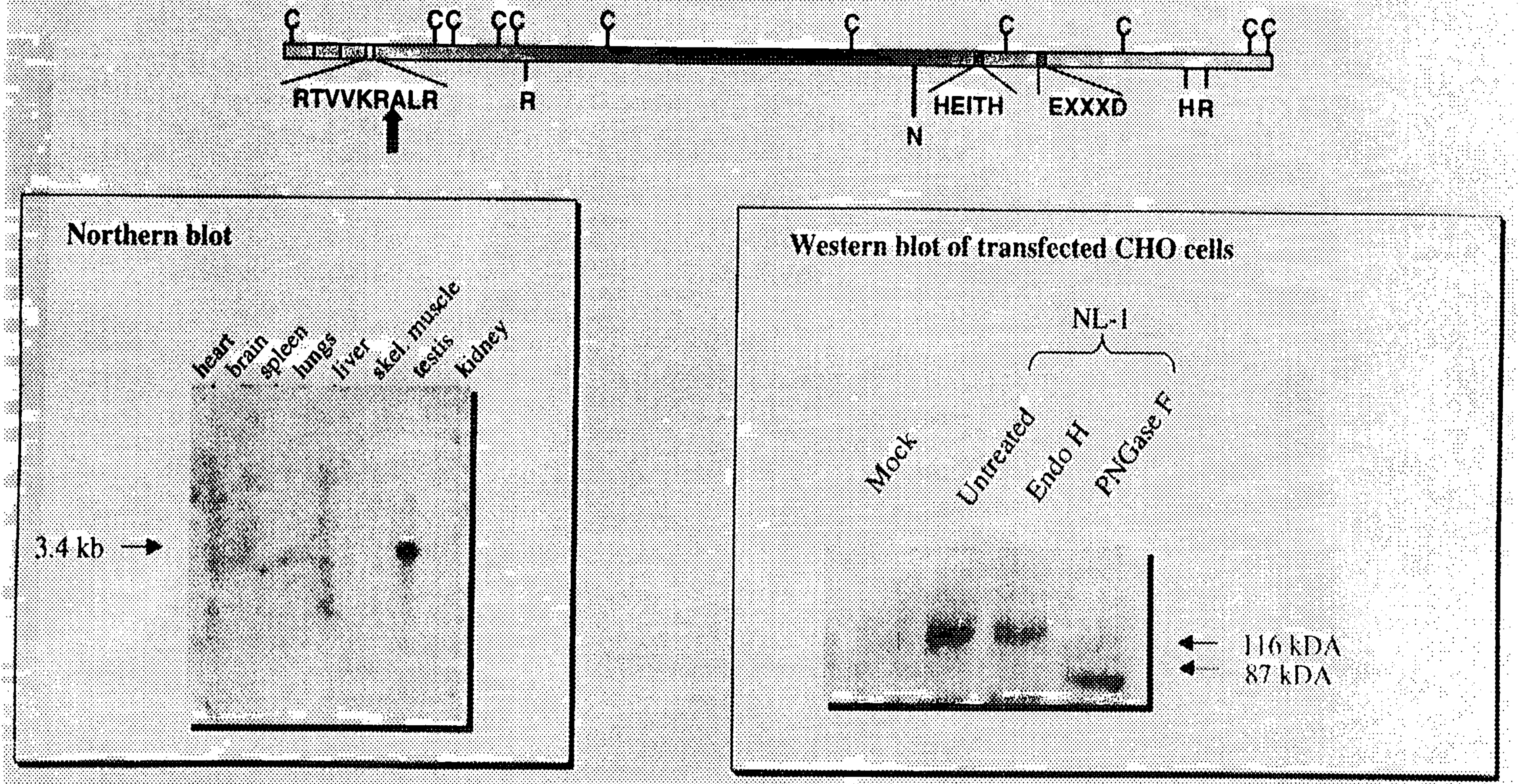


FIG. 10

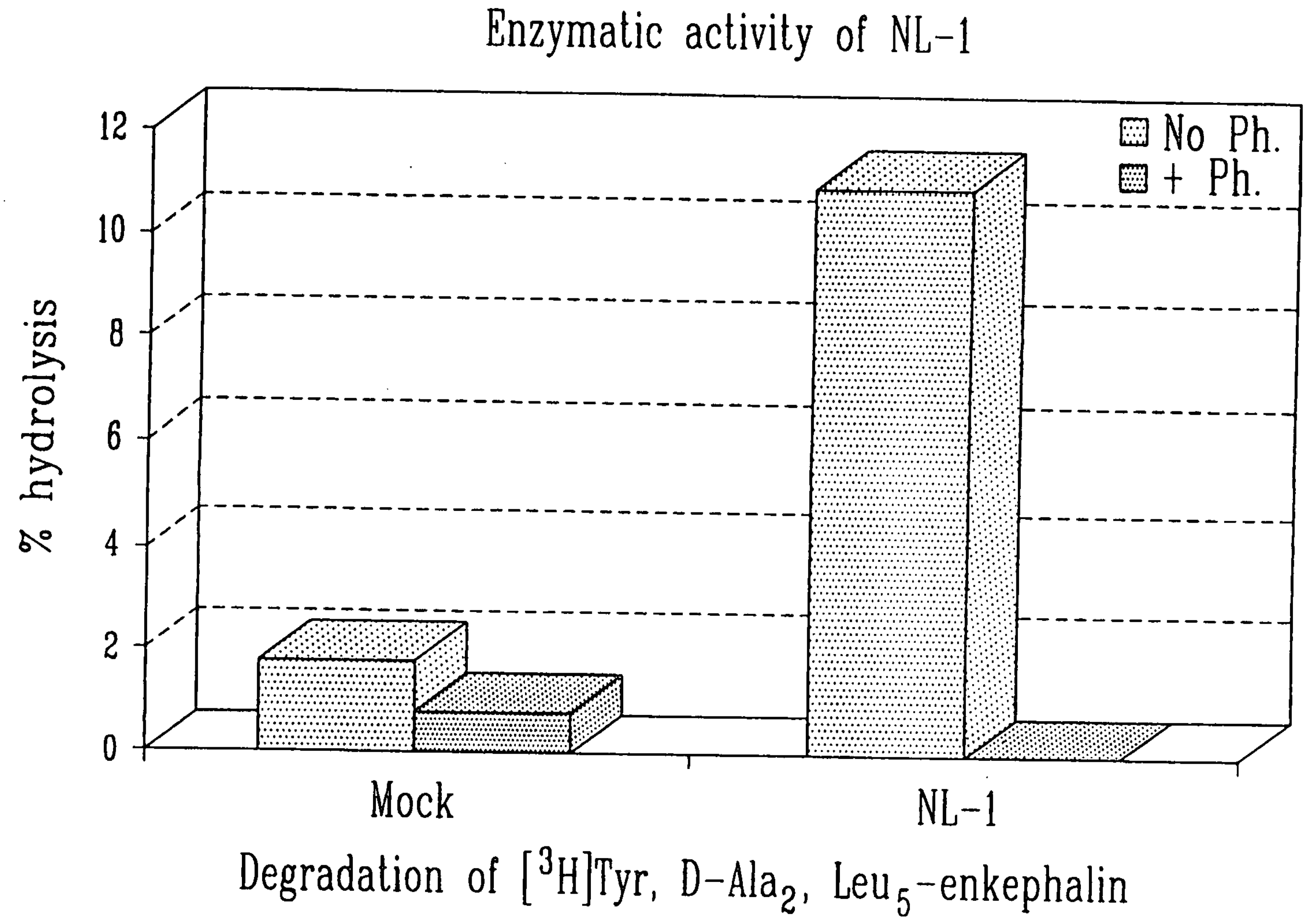

Enzymatic activity of NL-1
% hydrolysis
12
10
8
6
4
2
0
▨ No Ph.
▩ + Ph.
Mock
NL-1
Degradation of [^{3}H]Tyr, D-Ala$_2$, Leu$_5$-enkephalin

FIG. 11

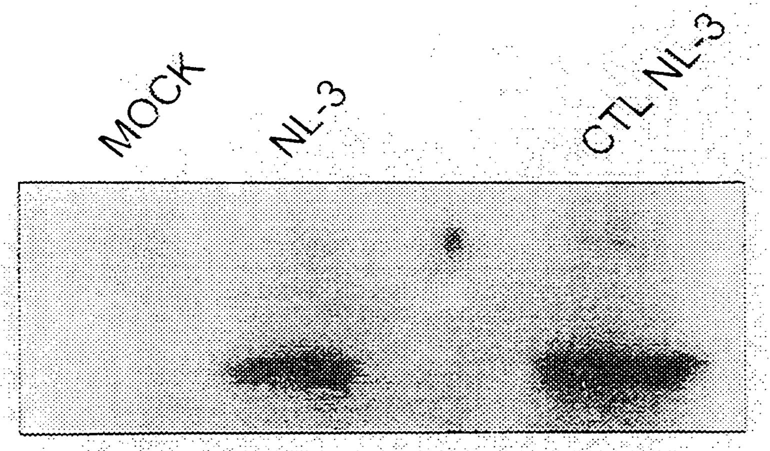


FIG. 12

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Published:
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— *With amended claims.*

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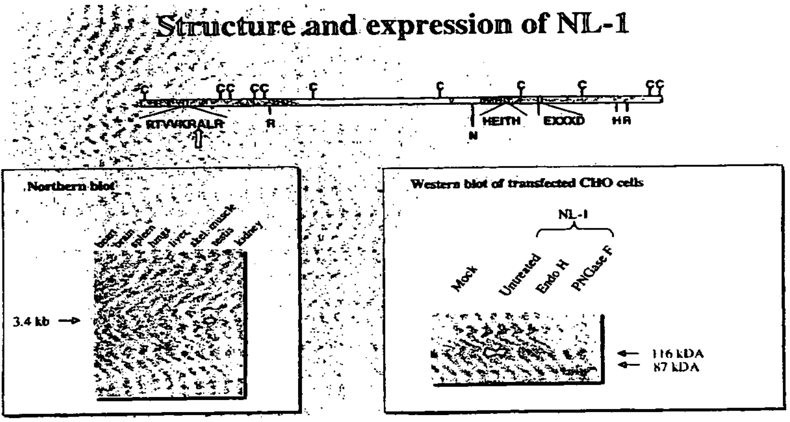
[Continued on next page]

(54) Title: METALLOPROTEASES OF THE NEPRILYSIN FAMILY



(57) Abstract: In this paper, we describe RT-PCR strategies that allowed us to identify and clone members of the NEP-like family. Degenerate oligoncleotide primers corresponding to consensus sequences located on either side of the HEXXH consensus sequence for zincins were designed and used in RT-PCR with mouse and human testis cDNAs. DNA fragments with lengths expected from the sequence of this class of enzympes were obtained. These DNA fragments were cloned and sequenced. Using this PCR strategy and the PCR fragments as probes to screen cDNA libraries, three zincin-like peptidases were identified in addition of known members of the family. The cDNA sequences allowed to derive specific probes for Northern and *in situ* hybridization, and probe human chromosomes to localize the gene and establish potential links to genetic diseases. Furthermore, these cDNA sequences were used to produce recombinant fusion proteins in *Escherichia coli* in order to raise specific antibodies. Finally, the cDNA sequences were cloned in mammalian expression vectors and transfected in various mammalian cell lines to produce active recombinant enzymes suitable for testing specific inhibitors.

WO 00/47750 A3

Date of publication of the amended claims: 25 May 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

AMENDED CLAIMS

[received by the International Bureau on 28 November 2000 (28.11.00);
new claims 23-27 added; remaining claims unchanged (4 pages)]

1. A neutral endopeptidase-like metallopeptidase named NL-1 which is isolated by probing tissue nucleic acids with degenerate oligonucleotides derived from a conserved sequence located on either side of a sequence His-Glu-Xaa-Xaa-His, wherein Xaa is any amino acid, which has a sequence selected from the amino acid sequences shown in Figures 3, 4, and 5, and a variant thereof sharing about 80% homology with said sequence.

2. A metallopeptidase as defined in claim 1, which has the amino acid sequence shown in Figure 3.

3. A metallopeptidase as defined in claim 1, which has the amino acid sequence shown in Figure 4.

4. A metallopeptidase as defined in claim 1, which has the amino acid sequence shown in Figure 5.

5. A nucleic acid encoding the metallopeptidase of any one of claims 1 to 3.

6. A recombinant vector comprising the nucleic acid defined in claim 4.

7. A recombinant host expressing the nucleic acid of claim 4.

8. A method for producing a metallopeptidase as defined in any one of claims 1 to 3, which comprises the step of culturing a recombinant host as defined in claim 6 in growth supportive medium and recovering said metallopeptidase from the cell or the culture medium.

\- A method for screening new molecules related to neural endopeptides, which comprises the step of:

- aligning nucleotidic sequences of known molecules related to NEP;
- assessing consensus sequences on either side of a sequence comprising His-Glu-Xaa-Xaa-His sequence;
- synthetising degenerate sequences of said consensus sequences;

- contacting said degenerate sequences with the nucleic acids of panel of candidate samples susceptible to express said new molecules, in conditions such that a hybridization complex can form between the nucleic acids of samples and the degenerate sequences;
- detecting said hybridization complex as an indication of a candidate sample which comprises a molecules related to NEP;
- sequencing the nucleic acids of said hybridized complex;

whereby a new sequence sharing homology with NEP is a new molecule relate to NEP.

10. A method as defined in claim 8 wherein said degenerate sequences are selected from Figure 2.

11. A method for producing a soluble form of a membrane protein of interest having a C-terminal ectodomain, said soluble form essentially consisting a said ectodomain, which comprises:

- obtaining nucleic acids encoding essentially the ectodomain;
- fusing the nucleic acids in phase with an amino terminal fragment of NL-1 or NL-2 as defined in C;
- having the fused nucleic acids to be expressed in a host cell in the presence of a culture medium, which expresses or is made to express furin;
- recovering said soluble form in the culture medium.

12. A method as defined in claim 9, wherein said protein of interest is NL-3 or β-endorphin.

13. An oligonucleotide selected from those in Figure 2.

14. A composition of matter comprising one or more of those in Figure 2.

15. A recombinant vector comprising a nucleic acid encoding the N-terminal part of the amino acid sequence shown in Figure 3 or 4 which N-terminal part terminates with a furin-recognition sequence.

16. A host transformed with the recombinant vector of claim 13.

17. The soluble form of the metallopeptidase defined in any one of claims 1 to 3 which soluble form essentially consist of the ectodomain of said metallopeptidase.

18. A composition comprising the soluble metallopeptidase of claim 15.

19 An oligonucleotide derived from the nucleic acid defined in claim 4, which oligonucleotide has at least 12 nucleic acid in length.

20. An antibody directed against the metallopeptidase defined in any one of claims 1 to 3.

21. A method for detecting the presence of the metallopeptidase as defined in any one of claims 1 to 3 in a sample, which comprises the steps of contacting: contacting said sample with the antibody defined in claim 18, in conditions such that an immune complex is formed between said antibody and said metallopeptidase, and detecting the presence of an immune complex as an indication of the presence of said metallopeptidase in said sample.

22. A method of detecting the presence or amount of the metallopeptidase as defined any one of claims 1 to 3 in a sample, which comprises the steps of: contacting said sample with the nucleic acid defined in claim 4 or with an oligonucleotide as defined in claim 17 in conditions such that a hybridization complex is formed between the target nucleic acids of the sample and the nucleic acids or oligonucleotides encoding said metallopeptidase, and detecting the formation of such hybridization complex as an indication of the presence of said metallopeptidase in said sample.

23. A method for obtaining an inhibitor of a neutral endopeptidase-like enzyme (NEP-like), which comprises the steps of :

-- contacting NEP-like with a molecule or extract in the presence of a NEP-like substrate; and
-- assaying the resulting solution for the intact substrate or for a decrease in the hydrolysed substrate as an indication of the presence of said inhibitor.

24. A method as described in claim 23, wherein said NEP-like has an amino acid sequence chosen from the amino acid sequences shown in Figures 3, 4 and 5 or a variant or fragment thereof.

25. A method as described in claim 23 or 24, wherein said NEP-like substrate is Tyrosyl-[3,5-^{3}H])(D-Ala$_2$)-Leu$_5$-enkephalin or bradykinin.

26. A method as described in any one of claims 23 to 25, wherein said assaying is realised with specific antibodies, HPLC or by the appearance of fluorescence when a self-quenched fluorescence tagged peptide is used as said NEP-like substrate.

27. A method as defined in any one of claims 23 to 26, wherein said molecule or extract is selected from identified synthetic libraries, biota extracts and from rationally designed inhibitors using X-ray crystallography and substituent activity relationships.